Clinical studies on hepatitis B, C, and E virus infection

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Clinical Studies on Hepatitis B, C, and E Virus Infection

Sophie Willemse
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The titan Prometheus created men from clay and stole fire from Zeus to comfort humanity. He was punished by Zeus for this act by being bound to a rock and have an eagle (Ethon) feed on his liver each day. During the night, Prometheus’ liver would grow back, to then have it eaten again next day, making the punishment one of perpetual suffering. Zeus then punished humanity by sending Pandora, and her box, unleashing disasters. In the end, Prometheus was freed by the hero Hercules. Prometheus’ persona symbolises audaciousness, inventiveness and perseverance; traits that are essential for every researcher.
Clinical Studies on Hepatitis B, C, and E Virus Infection

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General Introduction and Outline of the Thesis
GENERAL INTRODUCTION

HEPATITIS B VIRUS INFECTION

Virus and epidemiology
The hepatitis B virus (HBV) is an enveloped double-stranded DNA virus and it belongs to the family of Hepadnaviridae. Worldwide, there are over 350 million people chronically infected with HBV, with the highest prevalence in South-East Asia and Africa. As much as one third of the global population has encountered HBV-infection at some point in their life. Every year HBV-infection is responsible for over 780,000 deaths. Transmission of HBV may occur via different routes, being sexually, vertically (perinatal), and via blood-blood-contact. Perinatal transmission will lead to chronic HBV-infection in the majority of infected infants (> 95 %). When an acute HBV-infection is encountered later in life, the virus will be spontaneously cleared in most cases, but the chance of chronicity increases again in individuals who are infected with HBV > 65 years. Approximately 5 – 10 % of immunocompetent adult patients fail to clear the virus and become chronically infected with HBV. Patients with chronic HBV-infection (CHB) are at risk to develop liver cirrhosis (5-year cumulative incidence of 8 – 20 %) and hepatocellular carcinoma (HCC) (annual risk 2 – 5 %).

The prevalence of chronic HBV-infection in The Netherlands is 0.2 – 0.4 % (34,000 – 68,000 individuals). Serological testing in 1995 – 1996 has shown that 2.1 % of the population has ever had contact with the HBV. In 2006, the incidence of acute HBV-infection was highest in the Amsterdam and Rotterdam regions of The Netherlands (3.9 and 3.6 per 100,000 respectively). The overall incidence in The Netherlands in 2016 was 0.6 per 100,000. The prevalence and incidence of acute and chronic HBV-infection is higher...
among migrants from high-prevalence countries and individuals with risk behaviour (i.e. past injecting drug use, multiple sexual partners, male homosexual activity (MSM)).

Phases of HBV-infection
During CHB, multiple phases are distinguishable, which not always occur sequentially (Figure 2):7

– The HBeAg-positive immune-tolerant phase is the first phase of CHB. It is characterised by presence of hepatitis B e antigen (HBeAg), high HBV-DNA levels, but normal inflammatory parameters (serum alanine transferase (ALT), necro-inflammation on liver biopsy). This might last for decades, especially in subjects infected during the early years of life. There is no, or slow, progression of liver fibrosis.16

– The HBeAg-positive immune-reactive phase is the next phase of CHB, which is characterised by high or fluctuating inflammatory activity (reflected by elevated serum ALT) causing liver damage (possibly leading to fibrosis and cirrhosis) and a decrease in HBV-DNA levels. HBeAg-seroconversion might occur, resulting in the next phase.

– The HBeAg-negative inactive HBV carrier state (or low-replicative phase) follows the immune-reactive phase, when HBeAg is cleared and anti-hepatitis B e (anti-HBe) antibodies have been formed. HBV-DNA is low (< 2,000 IU/mL) and there is low inflammatory activity (reflected by serum ALT and no necro-inflammation on liver biopsy) resulting in minimal liver damage in most patients. HBsAg-loss and seroconversion to anti-HBs antibody might occur spontaneously in 1–3% of cases per year, usually after several years with persistently undetectable HBV-DNA.17

– The HBeAg-negative CHB phase may follow HBeAg-seroconversion during the immune reactive phase or may develop from the inactive carrier state.16 This phase is characterised by fluctuating high HBV-DNA levels (> 2,000 IU/mL) and inflammatory activity (reflected by elevated serum ALT and necro-inflammation in liver biopsy), leading to progressive liver damage (and, again, possibly to liver cirrhosis). Spontaneous clearance of hepatitis B surface antigen (HBsAg) and seroconversion to anti-hepatitis B surface (anti-HBs) antibodies, also named functional cure, might occur in a limited percentage of patients (1–3% per year).18, 19

– During the HBsAg-negative phase, low-level HBV-replication may persist with detectable HBV-DNA in liver, but not in serum (or in very low levels, <200 IU/mL), and presence of anti-HBc antibodies with or without anti-HBs.20 Immunosuppression may lead to reactivation in these individuals.21 If HBsAg-loss occurs before the development of cirrhosis, the outcome is improved with reduced risk of cirrhosis, decompensation and HCC. However, if cirrhosis has developed before HBsAg-clearance (13%), patients remain at risk of HCC (3%) and decompensation (14%), especially if there are concomitant liver diseases.22

Approximately 70% of all CHB patients have HBeAg-negative CHB with minimal inflammatory activity.21
Immune reaction in HBV-infection
To eliminate HBV after an acute infection, both the innate and the adaptive immune system are significant. The innate immune system is needed for early containment of the virus and initial activation of adaptive immune responses by producing interleukin 6 (IL-6) and type I interferon (IFN-I). IL-6 activates innate effector molecules, and IFN-I has direct antiviral effects, improves antigen presentation and activates natural killer T-cells (NK-cells). NK-cells have a direct antiviral effect by producing interferon-gamma (IFN-y). NK-cells can also modulate T-cell responses, which is important for lysis of virus-infected hepatocytes. When chronic infection is established, a strong adaptive immune response, primed by the innate immune response, is needed for viral clearance.

NK-cells are activated during acute infection. Thereafter, functionally active HBV-specific T-cells can be detected. In most individuals with acute resolving HBV-infection, strong and broad virus specific T-cell responses directed against the HBV-infected hepatocytes can be detected. However, these responses are weak and narrowly focused in patients who develop chronic HBV-infection, resulting in low levels of antiviral cytokines and attenuated cytotoxic T-lymphocyte (CTL) activity.

During chronic infection, HBV-specific T-cells are exhausted and their function is impaired. Multiple mechanisms are thought to be responsible for chronicity of HBV-infection. Both the innate and the adaptive immune system may prevent liver damage by inducing a so-called immune-tolerant state. In this phase, HBV-specific CD8 T-cells are deleted by NK-cells and cytotoxic T-cells are deviated to a suppressive or regulatory phenotype by priming through non-professional antigen-presenting cells (APCs). This attenuates the immune reaction which then is needed to clear the virus. However, it is still possible to induce an adequate immune reaction by IFN-I. On the other hand, HBV has the ability to avoid the development of an effective immune response by inhibiting adequate activation of the innate immune system. This results in an immune tolerant state, hampering viral clearance. A further mechanism by which viral clearance is disturbed, is T-cell exhaustion, which is thought to be caused by repetitive T-cell receptor stimulation by high HBsAg-levels, and aggravated by a lack of signalling from T-helper cells and by unfavourable cytokines such as interleukin-10 or arginase. However, whether HBV-specific T-cells are functionally active during the initial phases of infection is still unknown.

Various markers have been implicated to play a role in viral clearance of HBV and prediction of response to antiviral treatment. Amongst those are pre- or on-treatment HBsAg-levels and different cytokines or chemokines such as arginase, interleukin 10 (IL-10) and interferon-gamma-inducible protein 10 (IP-10). Levels of IP-10 are higher in CHB patients than in healthy controls. In CHB patients, serum IP-10 is positively correlated with serum HBV-DNA, serum ALT-levels, and progressive liver disease. Moreover, high pre-treatment serum IP-10 levels have been associated with HBeAg-loss during or after treatment with peginterferon (pegIFN) or nucleos(t) ide analogues (NAs).

**Treatment of chronic HBV-infection**

The goal of antiviral treatment in patients with CHB is to prevent progression to cirrhosis, hepatic decompensation and HCC by reducing viral replication and/or loosing HBsAg, (functional cure). Following the current treatment guidelines, the indications for treatment of CHB are generally the same for HBeAg-positive and HBeAg-negative patients, and are mainly based on serum HBV-DNA levels, serum ALT-levels and severity of liver disease. Treatment with antiviral therapy should be considered in CHB patients with the following characteristics:

- HBV-DNA levels above 2,000 IU/mL, ALT above the upper limit of normal (ULN), and severity of liver disease assessment showing at least moderate or severe active necro-inflammation (liver biopsy) or moderate liver fibrosis (liver biopsy/fibroscan) using a standardised scoring system such as HAI, Ishak or METAVIR;

- Liver cirrhosis with any detectable HBV-DNA and regardless of ALT-levels;

- HBV-DNA > 20,000 IU/mL and ALT > 2x ULN regardless of degree of fibrosis;

- Family history of HCC or cirrhosis and extrahepatic manifestations;
– Patients > 30 years who are still in the immune-tolerant phase with persistently high HBV-DNA and normal ALT-levels, regardless of degree of liver fibrosis.

For CHB patients with a low viral load, (HBV-DNA < 2,000 IU/mL) and no signs of necro-inflammatory activity or fibrosis, there is currently no indication for treatment, as viral replication is already low, and the progression of liver disease seems slow. However, CHB patients with a low viral load are at risk for the development of cirrhosis and HCC. As was shown in earlier cohorts, the risks for developing cirrhosis or HCC are highest in patients with the highest viral load (HBV-DNA > 200,000 IU/mL), however are already increased in those with a low viral load (HBV-DNA < 2,000 IU/mL). Furthermore, CHB patients with a low viral load can progress to HBeAg-negative chronic hepatitis, which could lead to fulminant hepatitis or cirrhosis. The threshold for starting anti-viral therapy based on HBV load has therefore been lowered to >2000 IU/mL in the current treatment guidelines compared to 20,000 IU/mL in the older treatment guidelines.

There are three different types of responses to therapy in CHB patients:

– Functional cure, defined as: HBsAg-loss with (HBs-seroconversion) or without formation of anti-HBs antibodies;

– Combined response, defined as: HBeAg-loss, low HBV-DNA (<2,000 IU/mL) and normal ALT in HBeAg-positive CHB patients; or low HBV-DNA < 2,000 IU/mL) and normal ALT in HBeAg-negative CHB patients;

– No HBeAg-loss, but low HBV-DNA (<2,000 IU/mL) and normal ALT in HBeAg-positive CHB patients.

Currently available treatment options for CHB are nucleo(s)tide analogues (NAs) and peginterferon (pegIFN). NAs inhibit HBV-DNA synthesis by competitive interaction with the natural substrates of the HBV-polymerase. The compounds registered in The Netherlands are: adefovir, tenofovir disoproxil and tenofovir alafenamide (all nucleotide analogues), lamivudine, telbivudine and entecavir (all nucleoside analogues) and emtricitabine (nucleotide analogue, prescribed in combination with tenofovir for HIV/HBV co-infection). All NAs are to be taken by a single oral dose. Because of their low level of resistance (in contrast to the other NAs), tenofovir and entecavir are the treatment options of first choice amongst the various NAs. The advantages of NAs are their effectiveness in suppressing HBV-replication and their good safety profile. Adequate viral suppression defined as undetectable levels of HBV-DNA in serum is achieved in >90% of patients treated with tenofovir or entecavir for 3 years. Long-term viral suppression improves clinical outcome in CHB patients, in terms of progression to cirrhosis, hepatic decompensation and development of HCC. Recent studies have shown that longstanding treatment with NAs can result in a reversion of liver fibrosis or even cirrhosis. NAs only inhibit HBV production, and not the production of viral proteins. Therefore, treatment with NAs rarely leads to loss of HBsAg. Stopping treatment would lead to recurrence of HBV-DNA in most cases, and long-term, usually life-long, treatment is therefore required.
Treatment with pegIFN on the other hand, is of finite duration of 48 weeks with the goal to induce maintained immunological control. PegIFN is administered subcutaneously once per week and has direct antiviral, but mostly immune modulatory effects. The applicability of treatment with pegIFN is generally poor as a result of its many side-effects (such as flu-like symptoms, hematological disorders, and depression), and a relatively low rate of response of 20–30% (HBeAg-seroconversion in HBeAg-positive patients or sustained low HBV-DNA (< 2,000 IU/mL) in HBeAg-negative patients). However, in selected HBeAg-positive CHB patients with a combination of certain baseline characteristics, higher response rates (HBeAg-loss) of above 50% may be achieved. These baseline predictors of HBeAg-loss are: genotype A, lower HBV-DNA levels (< 2x10^8 IU/mL), high ALT levels (> 2 ULN), female sex, older age (> 30 years) and absence of previous pegIFN therapy.

The most favourable treatment outcome of CHB patients is a functional cure. This is rarely achieved with a finite course of pegIFN or long-term viral suppression with NAs: only for 0–3% in NA therapy and 4–12% for treatment with pegIFN for 48 weeks.

Theoretically, it would be interesting to combine treatment with pegIFN and NAs because of their effect on modulating the immune response and their additional potent antiviral effect. Earlier attempts using this approach, with different compounds (lamivudine, adefovir, tenofovir and pegIFN) and in different settings (dual finite therapy or pegIFN add-on) showed varying results. Combination of lamivudine and pegIFN showed no benefit compared to either of the two therapies alone in both HBeAg-positive and HBeAg-negative patients. Combination therapy with pegIFN and adefovir or tenofovir in CHB patients with high viral load showed higher response rates than monotherapy with either medicament alone (pegIFN or NAs). However, these differences were small and might not weigh up to the increase in side effects caused by the addition of pegIFN. To achieve a better sustained therapeutic effect of treatment, new antiviral agents need to be developed.

HEPATITIS C VIRUS INFECTION

Virus and epidemiology
The hepatitis C virus (HCV) is an enveloped single-stranded RNA virus belonging to the family of Flaviviridae. The viral genome contains a long open reading frame of around 3011 codons encoding three structural proteins (core, E1 and E2), an ion channel (p7), and six non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). There are seven different genotypes (1–7), differing by 30–35% over the whole genome, which are subdivided into various subtypes (a-k).

HCV-infection is a leading cause of chronic hepatitis affecting over 170 million people worldwide. After being exposed to HCV (via blood-blood exposure), a chronic infection develops in 75–80% of cases. Chronic hepatitis C (CHC) virus infection is characterised by liver inflammation due to pro-inflammatory cytokines and infiltration of specific and non-specific T-lymphocytes. This leads to liver fibrosis and could ultimately cause liver cirrhosis, HCC and death. There is a large geographical variation in prevalence of HCV-infection and in many countries the epidemiology is not well known. At the same time, HCV-related mortality continues to increase as the infected population ages and advances to late-stage liver disease. In The Netherlands, estimates on
The antibody prevalence of HCV-infection vary from 0.1 to 0.6%. The most recent and reliable nationwide estimate was 0.22% (0.07–0.37%) in Dutch inhabitants aged 15–79 years in 2009, incorporating prevalence data among different subpopulations, corresponding with about 28,000 adult individuals ever infected with HCV. The risk groups of individuals with a chronic HCV-infection are mainly (ex)-drug users or migrants; those at risk of a getting infected with a new (acute) HCV-infection are mainly MSM. This situation is different in The Netherlands compared to many other countries, where HCV transmission among people who inject drugs (PWID) is ongoing, due to the extensive preventive measures among drug users such as awareness and clean needle programs.

**Immune reaction in HCV-infection**

After infection with HCV, the innate immune system initiates a non-specific immune response through type I IFN, leading to the activation of the intracellular pathway, resulting in the induction of multiple IFN-stimulated genes (ISGs). Type I IFN has also immunomodulatory effects by activating and modulating the function of different kinds of leukocytes, including NK-cells, macrophages, dendritic cells and T-lymphocytes. This results in a strong specific CD4+/CD8+ T-cell response leading ideally to the clearance of the virus. In acute HCV-infection, patients with self-limited infection have significant T-cell responses compared to little or no responses in those who evolve to chronicity.

Many cytokines and chemokines have been identified to be involved in immune reactions in response to HCV-infection. Most of these cytokines are modulated by exogenous IFN and play a critical role in viral clearance. In CHC, baseline activation of the immune system tends to be lower prior to treatment with IFN-based therapy.

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**Figure 3. Life cycle of hepatitis C virus.** Adapted from: Asselah T and Marcellin P. Liver Int 2011. Reproduced with permission, © John Wiley and Sons.
in patients who develop a sustained virological response (SVR) compared to those who do not.\textsuperscript{103, 104} This difference of baseline activation of the immune system might be influenced by single nucleotide polymorphisms (SNPs) on chromosome 19 near the interleukin-28B gene (IL28B), encoding IFN-\gamma. IL28B gene polymorphisms are highly associated with treatment outcome in CHC patients treated with IFN-based therapy.\textsuperscript{105} The gene encoding IP-10 is an ISG\textsuperscript{106, 107} that is induced by IFN-\gamma and tumour necrosis factor (TNF) alpha. IP-10 is produced by different kinds of cells such as endothelial cells, fibroblasts, mesangial cells, monocytes, neutrophils and hepatocytes. After binding to its receptor CXCR3, IP-10 functions as a chemotactic cytokine for T-lymphocytes, monocytes and NK-cells, and induces adhesion of activated memory/effector T-cells.\textsuperscript{108} Serum levels of IP-10 are higher in CHC patients than in healthy controls.\textsuperscript{109}

The relation between IP-10, viral clearance and response to antiviral therapy has extensively been described in acute and chronic HCV-infection.\textsuperscript{110–118} Furthermore, it was shown in CHC patients that intrahepatic IP-10 mRNA expression and plasma IP-10 levels are correlated with one another.\textsuperscript{113, 117, 118}

**Treatment of chronic HCV-infection**

Successful treatment of CHC has been challenging for some time. The first antiviral treatment available was recombinant IFN-alpha, which was first described in 1986, before the discovery of the virus in 1989.\textsuperscript{119} Success rates of this treatment were 38\% (assessed using ALT-levels as HCV-RNA levels could not yet be measured at the time).\textsuperscript{120} Ribavirin (RBV) is a nucleoside analogue with a broad antiviral activity, of which the exact mechanism of action is unknown.\textsuperscript{121} The addition of RBV to IFN-alpha ameliorated success rates of treatment, and became the standard of care in 1998.\textsuperscript{122} Modification of IFN-alpha by adding a polyethylene glycol (peg) to the molecule increased the half-life, which resulted in a once weekly administration (compared to 3 times a week) and a higher success rate compared to standard IFN-alpha.\textsuperscript{123} From the beginning of this century, treatment with pegIFN and RBV for 24–48 weeks (depending on genotype) has been the standard of care with SVR rates of 50\% in genotype 1 and 4 after treatment for 48 weeks, and 80\% after treatment for 24 weeks in genotypes 2 and 3.\textsuperscript{124} This treatment has multiple side-effects such as flu-like symptoms, fatigue,
haematological disorders (anaemia, leukopenia, thrombocytopenia), depression, emotional instability, and auto-immune diseases. In 2012, the first direct acting antivirals (DAAs) became available, the protease inhibitors boceprevir and telaprevir, directed against NS3 non-structural protein of the HCV. These medicaments worked exclusively for genotype 1, ameliorating SVR rates to 75%. However, as they were added to the standard therapy of pegIFN and RBV, the amount of side effects increased substantially, and many patients had difficulties in completing a full course of the therapy, which was still 24–48 weeks.125

In 2014, more new potent all-oral DAAs were registered, resulting in very high cure rates for patients with all HCV genotypes, and without many side effects.126–135 There are three classes of DAAs, each targeting different HCV proteins (Figure 4).

The DAAs that are now European Medicines Agency (EMA) approved:

**Protease inhibitors (-evir)**
The first group of DAAs targets the NS3/4A protease, inhibiting the cleavage of the precursor HCV protein, from which individual HCV proteins are further derived. The older drugs telaprevir and boceprevir belong to this group (first generation protease inhibitors), but newer drugs in this category are glecaprevir, grazoprevir, paritaprevir, simeprevir and voxilaprevir (second generation protease inhibitors). They all are combined with at least one other DAA from a different group.

**NS5A inhibitors (-asvir)**
The second group of DAAs targets the NS5A protein, inhibiting viral assembly and replication. There are multiple drugs registered in this category: daclatasvir, elbasvir, ledipasvir, ombitasvir, pibrentasvir and velpatasvir. These drugs also all need to be combined with at least one drug from another group to achieve SVR.

**Polymerase inhibitors (-uvir)**
The third, and possibly most potent group of DAAs is targeted against the NS5B protein, inhibiting the production of new HCV genomes. The most prescribed NS5B inhibitor is sofosbuvir, which is mostly used in combination with other DAAs, but started off as combination therapy with only RBV for genotype 2, 3 and 4, as other DAAs were not yet registered for these genotypes. Another polymerase inhibitor is dasabuvir, which is only registered in combination with paritaprevir/ritonavir and ombitasvir.

In The Netherlands, DAAs were due to their high price initially only reimbursed for patients with severe liver fibrosis or cirrhosis. Later, in 2015, the Dutch government decided to reimburse treatment with IFN-free DAAs for all CHC patients. The recommendations in the current European HCV treatment guidelines are in line with this decision, stating that all patients with compensated and decompensated chronic liver disease due to HCV-infection should be treated.137 The Dutch guidelines follow the statements of the EASL.138 SVR rates with DAAs are 90–95% for all genotypes in non-cirrhotic patients and treatment-naive Child-Pugh A cirrhotic patients. Figure 5. shows the progression of treatment success over the years. Even when there is cirrhosis, treatment success is still high in most genotypes. However, sometimes longer
treatment or the addition of RBV is advised. Whereas in the past, genotype 3 was one of the “easier” curable genotypes, it is now relatively more difficult to cure, as, especially in treatment-experienced cirrhotic patients, SVR-rates are around 80 – 86%.

Because of the low toxicity profile of many of the DAAs (except from protease inhibitors), even decompensated cirrhotic patients can be safely treated. Moreover, there are multiple regimens possible for patients with renal insufficiency, and treatment may also be given after renal transplantation as pegIFN is no longer required. The remaining questions regarding treatment with DAAs are directed towards DAA failure (mostly genotype 3), and resistance-associated substitutions (RAS), interactions with co-medication, HCC occurrence and recurrence after successful treatment, and the timing of treatment in decompensated cirrhotic patients who are listed for liver transplantation. Some small special groups still require attention, such as patients with short bowel, gastric bypass surgery, patients with low compliance, and patients with acute HCV-infection.

**HEPATITIS E VIRUS INFECTION**

**Virus and epidemiology**

Hepatitis E virus (HEV) is a non-enveloped, single-stranded RNA virus, containing three open reading frames (ORF1, ORF2 and ORF3). HEV can be subdivided into at least four genotypes. Genotypes 1 and 2 are human viruses causing acute hepatitis, mainly in young adults in tropical countries. Genotypes 3 and 4 are zoonotic, with pigs as the main reservoir in Europe and parts of Asia. The transmission routes of HEV genotypes 3 and 4 remain unclear, but it has been suggested that the consumption of undercooked meat products, contaminated with HEV, plays an important role. The most prevalent HEV genotype in Europe is genotype 3. After years of decline, the prevalence of HEV-infection in Europe is rising. Studies demonstrated that HEV-infection is widespread among blood donors, with incidences of viraemia varying from 1/762 in The Netherlands to 1 per 2,848 in the United Kingdom, 1 per 3,333 in Spain, and 1 per 8,416 in Austria. HEV-infection among donors is less prevalent in the United States (1 per 9,500).
**Immune reaction in HEV-infection**

After infection with HEV, a prominent antibody response is observed, directed against immunodominant antigenic epitopes in the ORF1, ORF2 and ORF3 of HEV. Early after infection an IgM anti-HEV response appears, followed by anti-HEV IgG a few days later. IgM disappears after 4–5 months, whereas IgG may persist for years to come. HEV-specific antibodies IgM and IgG seem to have a neutralizing function as their titers are shown to be significantly higher in patients with fulminant liver failure (FLF) due to acute HEV-infection than in those without FLF. In immunocompetent individuals, the diagnosis of HEV-infection is based on detection of anti-HEV IgG and IgM antibodies, because in most cases HEV IgM antibodies are detectable as soon as symptoms occur. However, in immunocompromised individuals, the diagnosis of HEV-infection must be based on detection of HEV-RNA, because those patients may remain seronegative, whereas HEV-RNA is detectable during chronic infection. Regarding the cellular immune response to HEV-infection, a robust non-specific IFN-γ production directed against the ORF1 and ORF2 region of the viral genome is described. Furthermore, in acute resolving HEV-infection, strong and multi-specific HEV-specific T-cell responses are observed, whereas in patients with chronic HEV-infection, HEV-specific CD4+ and CD8+ T-cell responses are rather weak.

![Figure 6. Proposed life cycle of hepatitis E virus. Adapted from: Cao D and Meng XJ. Emerg Microbes Infect 2012. Reproduced with permission, Copyright Nature Publishing Group.](image-url)
HEV genotype 3 in immunocompromised patients

Typically, HEV genotype 3 infection is asymptomatic and self-limiting. If present, symptoms include anorexia, nausea, fatigue, myalgia and jaundice with elevated bilirubin and liver enzymes. Furthermore, extrahepatic manifestations have been described, such as neurological manifestations (i.e. Guillain Barré syndrome\(^{168}\)), renal impairment, pancreatitis, cryoglobulinaemia and haematological abnormalities.\(^{169}\) However, HEV genotype 3 does pose a threat to immunocompromised patients who may develop chronic HEV-infection (58–93%)\(^{170–173}\) and liver cirrhosis.\(^{171}, 174, 175\) This was shown in different groups of immunocompromised patients such as solid organ transplanted (SOT) patients\(^ {170–173}, 175, 176\) and HIV-positive patients.\(^ {174}, 177\) In a Dutch cohort of 328 allogeneic hematopoietic stem cell transplantation (alloHSCT) recipients, 8 cases of HEV-infection (2.4%) were found of which 5 developed chronic hepatitis.\(^ {178}\) This suggests that there is a considerable risk of post-transplant HEV-infection for alloHSCT recipients. Most patients with HEV-infection have increased ALT-levels,\(^ {179}\) and most alloHSCT patients experience one or more episodes of elevated transaminase levels post-transplantation. However, the differential diagnosis of elevated liver enzymes following SOT or alloHSCT is extensive and includes medication toxicity, pre-existing liver conditions such as fatty liver disease, or graft versus host disease (GvHD) of the liver in case of alloHSCT.\(^ {180}\)

Treatment of chronic HEV-infection

Treatment options of chronic HEV-infection are: lowering of immunosuppressant therapy,\(^ {162}\) which is often dangerous and ill advised, or treatment with pegIFN\(^ {181}\) or RBV.\(^ {182}\) Due to the side-effects of pegIFN, RBV is the most frequently chosen treatment. RBV is often dosed as 600 mg per day, based on a case series described in 2014.\(^ {182}\) Most cases and case series describe a 3–6 month duration of treatment. A recent study showed an SVR of 63% after treatment for 3 months.\(^ {183}\) Therefore, in 2017, a dose of 1000–1200 mg per day rather than 600 mg per day is prescribed if tolerated. Protracted fecal shedding of HEV-RNA may predict treatment failure. Patients with chronic HEV-infection treated for 3 months with RBV who had still HEV-RNA detectable in their stools (excluding in plasma) all had a relapse after stopping therapy.\(^ {184}\)
OUTLINE OF THE THESIS

The following work describes various clinical aspects of viral hepatitis B, C, and E.

Part I of the thesis is focused on HBV-infection. In this part, different aspects of acute and chronic HBV-infection are addressed. Chapter 1 describes the immune responses of 9 patients with acute HBV-infection, in terms of NK-cell characteristics and HBV-specific T-cell function, with the aim to identify characteristics of patients who develop chronic HBV-infection versus those who spontaneously clear the virus. Chapter 2 aims to assess plasma- and intrahepatic IP-10 levels as a pre- and on-treatment marker of response to combination therapy with peginterferon (pegIFN) and adefovir in chronic HBV-infection. Chapter 3 describes a study in patients with chronic HBV-infection with low viral load who were randomised to be treated with a combination of pegIFN and adefovir, pegIFN and tenofovir or no treatment.

Part II of the thesis describes different aspects of chronic HCV-infection, with particular focus on treatment-related topics. In Chapter 4 the value of plasma IP-10 levels before and during treatment with high-dose induction interferon (IFN) is assessed for the prediction of treatment success. This is investigated in a previously described cohort of 85 chronic hepatitis C (CHC) patients (treatment naïve patients with various HCV genotypes who failed previous interferon-based therapy) who were treated for 6 weeks with high-dose IFN-alpha 2b (18 MU/day for 2 weeks, 9 MU/day for 2 weeks and 6 MU/day for 2 weeks consecutively), combined with ribavirin (RBV) (1000-1200 mg/day), followed by 24 or 48 weeks of pegIFN alpha 2b (1.5 ug/kg once a week) and RBV. Chapter 5 describes a retrospective multicentre study in the Amsterdam region of The Netherlands, evaluating the efficacy in real-life of combination treatment of sofosbuvir and simeprevir in CHC patients with genotype 4 with advanced liver fibrosis or compensated cirrhosis. In Chapter 6 a case is described of a CHC patient with HCV genotype 1b, who failed earlier antiviral treatment with a combination of two direct-acting antivirals (DAAs) (a protease- and NS5A-inhibitor), who, after undergoing gastric bypass surgery was successfully treated with a combination of sofosbuvir and simeprevir using therapeutic drug monitoring (TDM). Chapter 7 describes a prediction model to predict the future HCV disease burden in The Netherlands. In this chapter, a modelling approach is used to predict the effect of different treatment scenarios, in which various CHC patient groups are treated, on the size of the future HCV-viremic population and HCV-related disease burden.

Part III of the thesis is focused on HEV-infection, especially in immunocompromised patients as HEV-infection tends to become chronic in those patient groups. Chapter 8 assesses the prevalence of chronic HEV-infection in a cohort of 130 patients who underwent allogeneic hematopoietic stem cell transplantation (alloHSCT). The relation between HEV-infection and the occurrence of graft versus host disease (GvHD) in these patients was established.
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PART I

HEPATITIS B VIRUS INFECTION
CHAPTER 1

Dynamics of the Immune Response in Acute Hepatitis B Infection


In press Open forum for Infectious Diseases 2017
ABSTRACT

Background
Acute hepatitis B virus (AHB) infection in adults is generally self-limiting but may lead to chronicity in a minority of patients.

Methods
We included 9 patients with acute HBV infection and collected longitudinal follow-up samples. NK cell characteristics were analysed by flowcytometry. HBV-specific T cell function was analysed by in vitro stimulation with HBV peptide pools, and intracellular cytokine staining.

Results
Median baseline HBV DNA load was 5.12 log IU/mL and median ALT was 2,652 U/mL. Of 9 patients, 8 cleared HBsAg within 6 months whereas one patient became chronically infected. Early timepoints after infection showed increased CD56^{bright} NK cells, and an increased proportion of cells expressing activation markers. Most of these had normalised at week 24, while the proportion of TRAIL-positive CD56^{bright} NK cells remained high in the chronically infected patient. In patients that cleared HBV, functional HBV-specific CD8+ and CD4+ responses could be observed, whereas in the patient who developed chronic infection, only low HBV-specific T cell responses were observed.

Conclusions
NK cells are activated early in the course of acute HBV infection. Broad and multispecific T cell responses are observed in patients who clear acute HBV infection, but not in a patient who became chronically infected.
INTRODUCTION

Infection with the hepatitis B virus (HBV) affects large numbers of individuals worldwide. As much as one third of the global population has encountered HBV at some point in their life. Infection with HBV at early age will lead to chronicity in the majority of cases (> 95 %), resulting in an estimated 240 million patients worldwide who are chronically infected with HBV. When an acute HBV infection is encountered later in life, the virus will be spontaneously cleared in most cases. Less than 5 % of immunocompetent adult patients however, fail to clear the virus and become chronically infected with HBV. The mechanisms that lead to chronicity of hepatitis B infection are largely unknown. For clearance of the virus in the acute setting both the innate and the adaptive immune system are important. The innate immune system is responsible for early containment of the viruses and initial activation of adaptive immune responses. Although HBV has been shown to act as a ‘stealth’ virus in woodchucks and chimpanzees, evading early intrahepatic immune responses, it is uncertain whether these early innate responses are induced during acute HBV infection in man. Other players of the innate immune system, natural killer (NK) cells, are activated early during infection, before HBV-specific T cells arise. Later on during infection, functionally active HBV-specific T cells can be detected, which are thought to play an important role in viral clearance. In chimpanzees, depletion of CD8+ T cells at week 6 of infection lead to failure to clear the infection. During chronic infection, HBV-specific T cells are exhausted and their function is impaired. However, whether these HBV-specific T cells were functionally active during the initial phases of infection is unknown. In acute hepatitis C infection, patients with self-limited infection have significant T cell responses compared to little or no responses those who evolve to chronicity.

Acute hepatitis B infection is asymptomatic in the majority of cases which makes it difficult to study. However, previous studies have focused on blood donors that became HBsAg positive (n = 2) or a local outbreak (n = 5) for the initial phases of infection. Here we examined the early dynamics of NK and HBV-specific T cell responses in symptomatic patients with acute HBV infection who presented at our clinic.

PATIENTS AND METHODS

Patients

Patients were included at the gastroenterology and hepatology department of the Academic Medical Center in Amsterdam. Acute infection was diagnosed based on HBsAg and HBV DNA positivity, further serology, biochemistry and anamnesis reporting risk of acquiring HBV. Patients were assessed at the outpatient clinic at first visit (clinical onset) which was defined as baseline (BL). Follow-up was at week 1, 4, 12 and 24. All patients were HIV seronegative and were not co-infected with hepatitis C or hepatitis delta virus. The study was approved by the Ethical Review Board of the Academic Medical Center Amsterdam and all patients gave written informed consent. The study was conducted in accordance with Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements.
**Laboratory testing**

Biochemical and virological analyses were carried out by local laboratories in accordance with good laboratory practice. Qualitative detection of serum hepatitis B surface antigen (HBsAg) and antibody to hepatitis B surface antigen (anti-HBs) was performed by enzyme immunoassay (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). Quantitation of plasma HBV DNA levels was done by the Roche COBAS® TaqMan 48® assay (F. Hoffmann-La Roche Ltd, Diagnostics Division, Basel, Switzerland), with a dynamic range between 20 and \(1.70 \times 10^8\) IU/mL. HBV genotype was determined by sequencing a part of the polymerase gene with dideoxynucleotide technology.

**Sampling**

Peripheral blood samples were obtained at baseline (BL) and during follow-up (week 1, 4, 12 and 24). Sampling included plasma as well as peripheral blood mononuclear cells (PBMCs) which were isolated using standard density gradient centrifugation and subsequently cryopreserved until the day of analysis. Eight healthy blood donors were included for comparison.

**Cytokine measurements IP-10 and IL-18**

Levels of IP-10 and IL-18 were measured in available plasma samples with a DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). Values were extrapolated from standard curves (IP-10 range: 62.6–4000 pg/ml, IL-18 range: 11.7–750 pg/ml). Plasma samples from 6 healthy controls were included in the analyses.

**Immune phenotyping by flowcytometry**

PBMCs were washed in PBA (PBS containing 0.01 % (w/v) NaN3, 0.5 % (w/v) bovine serum albumin and 2 mM EDTA) and 1.0 \(\times\) 10^6 cells were incubated for 30 min in the dark at 4°C with different combinations of fluorescent label-conjugated mouse monoclonal antibodies (mAbs). For phenotypic analysis, the following mAbs were used: CD3 V500, CD56 BUV395, CD16 BV786, CD16 BV421, CD27 BUV737, HLA-DR FITC, CD38 PE-Cy7, PD-1 BV421, CD14 PE-CF594, CD19 PE-CF594 (BD Biosciences, San Jose, USA), CD8 BV711 CD8 BV785, CD57 Alexa Fluor 647 (Biolegend), life/dead fixable red stain (Invitrogen, Camarillo, USA), NKp46 PerCP-efluor 710, CD45RA efluor 605 (eBioscience, San Diego, USA), NKG2A PE (Beckman Coulter, Fullerton, CA, USA) and TRAIL APC (Miltenyi Biotec, Bergisch Gladbach, Germany). For intracellular staining, cells were fixed after surface staining with FACS Lysing Solution (BD) and subsequently permeabilized (FACS Permeabilizing Solution 2 (BD)). Cells were incubated for 30 min in the dark at 4°C with one or more of the following antibodies: perforin FITC (BD Biosciences), granzyme B PE (Sanquin, Amsterdam, The Netherlands), Ki67 BV711 (Biolegend, San Diego, CA, USA), Eomes PerCP-efluor 710, and T-bet Pe-Cy7 (eBioscience, San Diego, USA). Measurements were done using LSR Fortessa flow cytometer (BD Biosciences, Europe) and FACS Diva Software. Analysis was done using FlowJo v10 (FlowJo LLC, Ashland, USA).

**Intracellular staining HBV-specific T cells**

PBMC were cultured for 10 days with 5 pools of in total 315 15-mer peptides (10 overlapping residues) covering all proteins of HBV genotype A (Chiron Mimotopes, Victoria, Australia) at 1 µg/ml. PBMC were restimulated for 6 hours at day 10 in the presence of
CD107a PE (BD Biosciences, San Jose, CA, USA), Brefeldin A, and monensin. The production of cytokines was evaluated by intracellular staining with IFN-γ BV421, MIP-1β Pe-Cy7, TNF-α AF700, and IL-2 APC monoclonal antibodies (BD Biosciences, San Jose, CA, USA) after staining with surface markers as described above. Measurements and analyses were done as described above.

**Statistical analyses**
The two-tailed Mann-Whitney U test was used for analysis of differences between groups. For longitudinal analysis in individual patients the Wilcoxon signed rank test was used. P values < 0.05 were considered statistically significant. GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla, CA, USA) was used for analyses.

**RESULTS**

**Patients**
Nine patients with acute hepatitis B infection were included in the study (for baseline characteristics, see Table 1.). Patients were infected with genotype A (n = 5), or genotype B, D, E, or F (all n = 1). At BL, median HBV DNA load was 5.12 log IU/mL (iqr: 3.80–6.64, Figure 1A) and median ALT was 2,652 U/mL (iqr: 1,554–3,390 U/mL Figure 1B). Six months after infection, 8 of 9 patients had spontaneously cleared the virus, of which 6 had formation of anti-HBs antibodies. One patient, infected with HBV genotype A, did not clear HBsAg within 6 months. At 6 months after initial presentation the viral load in this patient was > 1.7x10⁸ IU/mL (upper limit of quantification) and ALT was 454 U/mL (Figure 1A,B). In a subset of patients, IP-10 and IL-18 levels were measured. At baseline, the plasma levels of IP-10 were increased in patients with acute HBV infection as compared to healthy controls (median 1613.0 and 39.4 pg/mL respectively, p = 0.0007, Figure 1C). In addition, baseline IL-18 levels were significantly increased in patients with AHB infection (median 1182 pg/mL) as compared to healthy controls (median 124.3 pg/ml, p=0.002, Figure 1D). At week 24, IP-10 levels had normalised, while IL-18 levels were still increased as compared to healthy controls (median 306.0 and 124.3 pg/mL respectively, p = 0.007 Figure 1D).

Early time points after infection (BL and week 1) showed an increase in the proportion of CD56bright NK cells (BL median 7.4 %) as compared to healthy controls (median 1.4 %, p = 0.0028, Figure 2A). Furthermore, the proportion of CD56dim NK cells was decreased (Figure 2B) early during infection, while the proportion of total NK and total CD8+ T cells was not significantly different from that of healthy controls (Figure 2C,D). There was no significant change in the proportion of effector and memory CD8+ T cells. However, the memory T cell population was activated, as demonstrated by the significant increase in PD-1, Ki67, HLA-DR/CD38, perforin and granzyme B positive memory T cells (Supplementary Fig 1).

**Early NK cell activation**
To investigate the role of NK cells at different time points during acute infection we measured the expression of several markers of NK cell activation. At baseline, the proportion of CD56bright NK cells expressing CD38 was significantly increased (median 44.0%) as
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Table 1. Baseline characteristics

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Figure 1. Course of HBV DNA (A), ALT (B), IP-10 (C) and IL-18 (D) in patients with acute HBV infection. Not all patient samples were available for IP-10 (n = 5) and IL-18 (n = 4) measurements. Statistical testing: Mann-Whitney U test. lloq; lower limit of quantification, uloq; upper limit of quantification; ns, non-significant; ***, p < 0.001; **, p < 0.01.
compared to healthy controls (median 30.3%, p = 0.01, Figure 3A). Similarly, CD38 was expressed on 56.2% of CD56^dim NK cells in AHB patients at baseline as compared to 42.0% of HC CD56^dim NK cells (p = 0.03, Figure 3A). At baseline, HLA-DR was expressed on an increased proportion of CD56^dim NK cells (median 18.3%) in AHB patients as compared to healthy controls (median 4.6%, p = 0.0008). The proportion of HLA-DR+ CD56^dim NK cells was still increased 1 week after presentation of AHB infection (Figure 3B). Ki67, a marker for proliferation, was not differentially expressed between baseline NK cells from AHB patients and healthy controls. At week 4 and week 12 however, the proportion of Ki67+ CD56^dim NK cells was increased as compared to healthy controls (Figure 3C). The differentiation status of NK cells, as measured by CD57 and NKG2A expression, was not different in patients with acute hepatitis B infection as compared to healthy controls (Supplementary Fig 2).

Figure 2. Proportion of CD56^bright (A) and CD56^dim (B) NK cells, total NK cells (C), and total CD8+ T cells (D) in 9 patients with acute HBV infection as well as in healthy controls (HC). Statistical testing: Mann-Whitney U test. ns, non-significant; **, p<0.01.
**Long term increase in effector markers**

The proportion of CD56\textsuperscript{bright} NK cells expressing NKp46, an activating receptor, was significantly increased at all time points during acute HBV infection, as compared to healthy controls (AHB baseline median 96% and HC 81.6%, p < 0.0001, Figure 4A). This was similar for CD56\textsuperscript{dim} NK cells (Figure 4A). The TNF related apoptosis inducing ligand TRAIL is generally expressed by a minority of CD56\textsuperscript{bright} NK cells. At baseline acute HBV

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**Figure 3.** Markers of NK cell activation CD38 (A), HLA-DR (B), Ki67 (C) in CD56\textsuperscript{bright} (left) and CD56\textsuperscript{dim} (right) NK cells. Statistical testing: Mann-Whitney U test. ns, non-significant; ***, p < 0.001; *, p < 0.05.
infection, the proportion of TRAIL+ CD56\textsuperscript{bright} NK cells was significantly increased as compared to healthy controls (median 11.7 and 2.2 \% respectively, \( p = 0.0028 \), Figure 4B). Furthermore, TRAIL expression in the patient that was chronically infected was still elevated at week 24 after initial presentation (29.5\% of CD56\textsuperscript{bright} NK cells, Figure 4B). During acute HBV infection the proportion of granzyme B and perforin positive CD56\textsuperscript{dim} NK cells was significantly increased compared to healthy controls (granzyme B baseline AHB 71.7 and HC 48.6 \%, \( p = 0.04 \). Perforin baseline AHB 82.4 \% and HC 63.3 \%, \( p = 0.01 \), Figure 4C,D). In addition, granzyme B was expressed by a considerate proportion of CD56\textsuperscript{bright} NK cells, which are generally thought to play a lesser role in cytotoxicity (Figure 4C). In particular, a high proportion of granzyme B expressing CD56\textsuperscript{bright} NK cells remained present in the patient who evolved to chronic infection (Figure 4C).

**HBV-specific T cell responses**

HBV-specific T cell responses, measured by cytokine production in response to HBV peptide pools, were analysed in 5 patients who were infected with HBV genotype A. Even though intra-individual differences were observed in the specificity and timing of the response, HBV-specific T cell responses could be observed in all patients that spontaneously cleared the HBV infection during the 6 months follow-up (Figure 5A-D). In the patient who became chronically infected however, very low HBV-specific T cell responses were observed at all time points (Figure 5E). Patients who cleared the HBV infection could have an early peak of the HBV-specific CD8+ response (patient 4 and 6) or a later peak (patient 5 and 8). The specificity of the T cell response also differed between patients. In patients 4 and 5 the highest observed sum of cytokine responses was observed in response to the polymerase peptide pool, whereas in patient 6 the highest cytokine production was against the envelope pool, and in patient 8 against the core peptide pool. CD4+ T cell responses mostly followed the same patterns (Supplementary Figure 3).

![Figure 4. Effector markers NKp46 (A), TRAIL (B), Granzyme B (C) and Perforin (D) in CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells. Statistical testing: Mann-Whitney U test. ns, non-significant; ****, \( p < 0.0001 \); ***, \( p < 0.001 \); **, \( p < 0.01 \); *, \( p < 0.05 \).](image-url)
Continuation of Figure 4.
Figure 5. HBV-specific CD8+ T cell functionality was assessed in patients who were infected with HBV genotype A. TNFα, IL-2, MIP-1β and IFNγ production by HBV-specific CD8+ T cells in 4 patients who cleared AHB infection (A-D) and 1 patient who evolved to chronicity (E).
DISCUSSION

Here we show the temporal dynamics of NK cells and T cells in nine patients during acute hepatitis B infection, of which one developed chronicity. HBV is a non-cytopathic virus, however, immune responses mounted by the host can cause serious liver damage. Whereas viral clearance can occur without clinical symptoms or cell destruction,4,11 in our patient cohort hepatocyte damage was apparent by ALT elevations. As we observed a significant increase in the proportion of total peripheral memory T cells expressing makers of activity and cytolytic proteins, HBV-nonspecific bystander T cells could have played a role in hepatocyte injury,16, 17 as seen in mouse models of acute fulminant HBV infection.18 At the time of presentation at the clinic, plasma levels of IP-10, an interferon stimulated gene (ISG), were significantly increased suggesting broad innate immune activation via the interferon pathway. Previous studies have shown no significant induction of type I interferons in the initial phases of acute HBV infection in chimpanzees8 or woodchucks.9
Also in man, type I interferon production was not observed early during infection,\textsuperscript{10} still, it has not been ruled out that other interferon types such as IFN-\lambda are induced.\textsuperscript{10} As we included patients who already were symptomatic, it is more likely that local danger signals or production of interferons by lymphocytes have activated the ISG pathway.\textsuperscript{4}

Early during the infection, the proportion of CD56\textsuperscript{bright} NK cells was significantly increased as compared to healthy controls. Furthermore, we observed a striking increase in the proportion of CD38, HLA-DR and Ki67 positive NK cells, indicating activation of these cells. Previously, it was shown that in patients with symptomatic infection, NK cell function in inhibited before peak viremia, and NK cells only become activated after peak viremia.\textsuperscript{10} As we only included patients after peak viremia, we may have missed the described inhibition of NK cells in the preclinical phase of infection. The proportion of NK cells expressing perforin and granzyme B was increased in patients with an acute HBV infection. While in mice, hepatocytes infected with a recombinant adenovirus have been suggested to be resistant to perforin mediated killing,\textsuperscript{19} the increase in the proportion of NK cells expressing cytotoxic molecules suggests they could have a role in hepatocyte damage. As such, an increase in perforin expression by HBV-specific T cells has been observed in acute HBV infection.\textsuperscript{20}

As the proportion of TRAIL expressing CD56\textsuperscript{bright} NK cells was significantly increased, TRAIL mediated killing of infected hepatocytes could also be responsible for ALT elevations.\textsuperscript{21} However, in two previously described patients who cleared the infection without any symptoms or ALT elevations, NK cells were activated early in the course of acute HBV infection, emphasizing that these cells can be important in non-cytopathic elimination of HBV.\textsuperscript{11} While Ki67, HLA-DR, and CD38 normalised after clearance of the virus, other NK cell markers were still increased at week 24 as compared to healthy controls, including Nkp46, TRAIL, perforin, and granzyme B. In line with this, baseline elevated levels of IL-18, which is associated with NK cell activation,\textsuperscript{22} were still significantly elevated at week 24. Interestingly, in patients with chronic hepatitis B, TRAIL expression is significantly elevated.\textsuperscript{23} Similarly, in the patient who evolved to chronic infection, TRAIL remained expressed on a significant proportion of CD56\textsuperscript{bright} NK cells. Whether this TRAIL positive population is a cause or a result of chronic infection remains unanswered. During chronic infection, TRAIL has been suggested to play a role in killing of infected hepatocytes,\textsuperscript{21} as well as in NK cell mediated clearance of HBV-specific T cells.\textsuperscript{24}

Even though HBV-specific T cells seem to be inhibited by IL-10 and arginase early during infection,\textsuperscript{10, 25} their presence is highly associated with viral clearance.\textsuperscript{5, 10, 26} From acute HCV infection, we know that evolution to chronicity is associated with weak and transient responses of HCV-specific T-cells.\textsuperscript{14, 27} Clearance of the infection on the other hand, is associated with the appearance of multi-specific HCV-specific CD8+ T-cell responses against multiple epitopes.\textsuperscript{27} However, in two blood donors with acute HBV infection who were followed during asymptomatic infection, HBV-specific T cell responses reached their peak when HBV DNA was already declining, emphasizing the importance of other immune mechanisms for antiviral activity.\textsuperscript{11} Early T cell responses in these patients were directed against envelope and polymerase proteins.\textsuperscript{11} In 2 other patients, early responses were directed against polymerase and the X protein, followed by responses against core and envelope.\textsuperscript{10} Here we observed broad reaction to all HBV proteins in 2 patients at the earliest time point (patient 4 and 6). A more delayed and
narrow HBV-specific T cell response was observed in 2 other patients, while they already showed ALT elevation and viral load decline (patient 5 and 8). Previously, a lack of T cell responses was associated with persistently high HBV DNA levels and the need for treatment in an immunosuppressed patient. In the one patient who did not clear HBV infection, the observed narrow T cell response may have led to chronic infection. This is in line with evolution of chronicity in woodchucks infected with the woodchuck hepatitis virus, as well as observations in acute hepatitis C patients.

Acute HBV infection can present in many different ways. With or without ALT elevations, and with or without symptoms. Therefore, the underlying mechanisms may also differ between cases. As one of the patients in this study developed chronic infection we had the unique opportunity to analyse early events that could be associated with failure to clear HBV. Our data suggests that the absence of a HBV-specific T cells response at all time points could play a role in progression to chronic infection. Furthermore a substantial population of CD56bright NK cells expressing TRAIL, as seen in chronic HBV infection, was present at all time points in this patient. A better understanding of the early course of acute infection and the evolution to chronicity may help the development of novel therapeutics targeting chronic hepatitis B virus infection.

**SUPPLEMENTARY DATA**
Supplementary data are available on request.
REFERENCES


CHAPTER 2

Intrahepatic IP-10 mRNA and Plasma IP-10 Levels as Response Marker for HBeAg-positive Chronic Hepatitis B Patients Treated with Peginterferon and Adefovir


Antiviral Research 2016; 131: 148–155
ABSTRACT

Introduction
Interferon-γ-inducible protein-10 (IP-10), also called CXCL10, is produced by different types of cells such as monocytes, neutrophils and hepatocytes. IP-10 functions as an inflammatory cytokine, which after binding to its receptor CXCR3, expressed on T-lymphocytes, leads to immune activation. We aimed to establish if IP-10 expression in liver tissue and in plasma of chronic hepatitis B (CHB) patients correlated with each other and further to investigate if IP-10 levels before and during therapy with peginterferon and adefovir could predict treatment outcome in CHB patients.

Patients and methods
A total of 86 CHB patients (41 HBeAg-positive and 45 HBeAg-negative) received combination therapy of peginterferon and adefovir for 48 weeks. Combined Response (CR) (HBeAg-negativity, HBV-DNA ≤ 2,000 IU/mL, ALT normalization) and non-response (NR) were assessed at Week 72. Plasma IP-10 levels were measured at baseline and during treatment at Day 3 (D3) and Week 1 (W1). Pre-treatment liver biopsies from 40 of 86 patients were obtained and stored in liquid nitrogen for the analysis of intrahepatic IP-10 mRNA expression.

Results
CR was achieved in 14/41 HBeAg-positive and 17/45 HBeAg-negative patients. Mean baseline plasma IP-10 levels were significantly higher in HBeAg-positive patients with CR than NR (3.20 vs 3.00 log pg/mL, p = 0.03); but not in HBeAg-negative patients. Baseline IP-10 levels correlated with ALT-levels in HBeAg-positive and -negative patients (both p < 0.001), and with a decline of HBsAg-levels of ≥ 0.5 log IU/mL at Week 12 in HBeAg-positive patients (p = 0.001). Plasma IP-10 levels were associated with intrahepatic IP-10 mRNA expression, however, more strongly in HBeAg-positive (R=0.79, p<0.001) than in HBeAg-negative patients (R=0.53, p = 0.011). IP-10 levels only correlated with HAI-scores in HBeAg-positive patients (R = 0.40, p = 0.025). Mean plasma IP-10 levels of both HBeAg-positive and -negative patients increased significantly at D3 compared to baseline (+0.30 log pg/mL, p = 0.003), to then decline subsequently at W1 to a level still significantly higher than baseline (+0.14 log pg/mL, p < 0.001). The increase of IP-10 was significantly higher in HBeAg-positive patients with NR than in those with CR (+0.35 versus +0.11 log pg/mL, p = 0.003).

Conclusions
Baseline plasma IP-10 levels and IP-10 mRNA expression in the liver are correlated with each other, suggesting that plasma IP-10 reflects intrahepatic immune activation. Higher IP-10 levels at baseline seem to be associated with CR in HBeAg-positive patients treated with peginterferon and adefovir, but not in HBeAg-negative patients.
INTRODUCTION

Worldwide, there are over 350 million people chronically infected with the hepatitis B virus (HBV), with the highest prevalence in South-East Asia and Africa.\(^1\) Every year HBV infection is responsible for over 780,000 deaths.\(^2\) In acute infection an adequate immune response and resolution of the virus with lifelong protective immunity is seen in approximately 95% of adult people. Perinatal transmission of HBV from mother to neonate has a higher chance to result in chronic infection.\(^3\) When the host immune response is inadequate and the infection persists, patients become chronically infected and are at risk to develop liver cirrhosis and hepatocellular carcinoma.\(^4\) The mechanism of developing chronic hepatitis B virus (HBV) infection, rather than clearing acute infection, is not fully understood. It has been associated with impairment of the innate and adaptive immune responses. In most individuals with acute HBV infection that spontaneously resolve, strong and broad virus specific T-cell responses directed against the HBV-infected hepatocytes can be detected. However, these responses are weak and narrowly focused in patients who develop chronic HBV infection, resulting in low levels of antiviral cytokines and attenuated cytotoxic T-lymphocyte (CTL) activity.\(^5\)–\(^12\) During chronic HBV infection inflammatory activity and HBV load may fluctuate in some patients whereas in others inflammation is absent and HBV load remains low.

Many cytokines and chemokines have been identified to be involved in immune reactions in response to HBV infection. Interferon-\(\gamma\)-inducible protein-10 kDa (IP-10/CXCL10) is a non-EXR-CXC chemokine\(^13,14\) and is a chemo-attractant for T-lymphocytes, monocytes, and NK-cells. This cytokine and especially its relation to viral clearance and response to antiviral therapy have extensively been described in acute and chronic hepatitis C virus (HCV) infection.\(^15\)–\(^24\) Next to this, it was shown in chronic HCV patients that intrahepatic IP-10 mRNA expression and plasma IP-10 levels are correlated with each other.\(^18,23,24\) In chronic HBV patients, serum IP-10 seems to be higher than in healthy controls.\(^25,26\) Serum IP-10 is positively correlated with HBV-DNA, ALT levels and progressive disease in chronic HBV infection.\(^8,27\)–\(^31\) Next to this, high pre-treatment IP-10 levels have been associated with HBeAg-loss during or after treatment with peginterferon\(^32\) or nucleo(s) tide analogues (NAs).\(^33\) Combination therapy with peginterferon and adefovir\(^34\) or tenofovir\(^35\) in active CHB patients showed higher response rates than monotherapy with either medicament alone (peginterferon or NAs).

It is not entirely clear whether IP-10 levels at baseline or changes in IP-10 levels during treatment predict response to treatment in active CHB patients, treated with a combination of peginterferon and a nucleo(s) tide analogue (NA). Aside from this, a correlation between intrahepatic IP-10 mRNA expression and plasma IP-10 levels in chronic HBV infection has not been investigated. Our aim was to establish if IP-10 levels at baseline and early during therapy can predict treatment outcome, and to establish if IP-10 mRNA expression in liver tissue is correlated with plasma IP-10 levels in CHB patients.
PATIENTS AND METHODS

Patients, Treatment regimen and Sample collection
From 2005–2008, an open label prospective study was performed in which 86 CHB patients with active CHB (HBV-DNA ≥ 20,000 IU/mL and ALT above upper limit of normal (45 U/L for males and 34 U/L for females) or histological signs of chronic active hepatitis) were included (41 HBeAg-positive and 45 HBeAg-negative patients). Inclusion of patients was done according to the Dutch national guidelines on the indication for treatment of chronic HBV-infection at the time. The results of this study were reported in 2013 (34). All patients were treated for 48 weeks with peginterferon-alpha-2a 180 µg subcutaneously once per week (Roche, Switzerland) and adefovir dipivoxil 10 mg daily (Gilead Sciences, USA). Plasma samples were obtained and stored at -80°C at baseline (BL), Day 3 (D3), Week 1 (W1), Week 18 (W18) during treatment, end of treatment (EOT) and at Week 24 after cessation of treatment (Week 72).

Plasma measurements
HBV-DNA was measured quantitatively at BL and at Week 72 using the Roche COBAS Taqman 48® assay (dynamic range 20–1.70 × 10E8 IU/ml, F. Hoffmann-La Roche Ltd, Diagnostics Division, Switzerland). HBsAg-levels were measured quantitatively at BL, Week 12 (W12) and Week 72 (W72) using the Abbott Architect (dynamic range 0.05–250 IU/mL, Abbott Diagnostics, USA).36 HBV genotype was assessed at BL using the INNO-LiPa-assay (Innogenetics, Gent, Belgium) or by sequencing a part of the polymerase gene with dideoxynucleotide technology. ALT, anti-HBs, HBeAg, anti-HBe were measured at BL by our local laboratory in accordance to good laboratory practice. Plasma IP-10 levels at baseline were measured using a Luminex polystyrene bead-based assay (Bio-Plex Pro, BioRad, Hercules CA, USA). Then, we performed longitudinal measurement of plasma IP-10 at BL, D3 and W1, using a solid base sandwich enzyme linked immunosorbent assay (sensitivity 4.46 pg/ml, assay range 7.8–500 pg/ml; Quantikine human CXCL10/IP-10 immunoassay, R&D Systems). Accordance of the test results between the two tests and time points was seen in 82 of 86 patients, so for the longitudinal analysis only these patients were included. Of these 82 patients, further longitudinal measurement of plasma IP-10 at W18 and at EOT was performed in 22 patients, of which 12 were HBeAg-positive and 10 HBeAg-negative.

Liver biopsy specimens and Measurements
Pre-treatment liver biopsy specimens were obtained in 69 of 86 patients. Of these specimens, 40 were stored in liquid nitrogen, enabling the analysis of intrahepatic IP-10 mRNA expression using an in-house developed reverse transcription quantitative polymerase chain reaction (RT-qPCR). First, three micrograms of RNA were reverse transcribed using M-MLV reverse transcriptase and random hexamer primers. Relative quantification of gene expression was determined with the LightCycler 480 Real-Time PCR System (Roche Applied Science, Rotkreuz, Switzerland) using the SYBR Green PCR Master Mix and for IP-10 gene specific primers. PCR product specificity was assessed by melting curve analysis. Thereafter, mRNA expression levels were normalized to the arithmetic mean of two housekeeping genes (ACTB and GUSB) using the comparative Ct method, and log_{10} transformed for analysis. All liver biopsy
specimens were assessed by an experienced pathologist on activity score using the modified Ishak histopathologic activity index (HAI)\textsuperscript{37, 38} and on fibrosis stage using the Ishak score.\textsuperscript{38}

**Assessment of treatment outcome**
Assessment of response to therapy was based upon HBsAg-loss, HBeAg-loss and HBV-DNA decline (≤2,000 IU/mL at W72). Responses were defined in accordance to the most recent AASLD and EASL guidelines.\textsuperscript{39, 40} HBeAg-loss was defined as undetectable HBeAg at W72, and HBeAg seroconversion was defined as HBeAg-loss with formation of anti-HBe at W72. HBsAg-loss was defined as undetectable HBsAg at W72, and HBsAg seroconversion as HBsAg-loss with formation of anti-HBs at W72. Combined response (CR) was defined as a combination of virological (HBeAg-negative and HBV-DNA ≤ 2,000 IU/mL) and biochemical response (persistent normal ALT values) in all patients. Patients were considered non-responder (NR) if they did not meet the definition for one or both criteria for CR, when there was a virological and/or biochemical relapse, or when re-treatment with a NA was started after end of therapy. Relapse was defined as HBV-DNA > 2,000 IU/mL (virological relapse) and/or ALT above upper limit of normal (biochemical relapse) after stopping therapy.

**Statistical analysis**
Levels of plasma IP-10, HBsAg and HBV-DNA were logarithmically transformed to achieve a normal distribution. Graphic representation was performed using Graphpad Prism version 5 and 6 for Windows\textsuperscript{®} (GraphPad Software Inc., San Diego, California, USA). Statistical comparisons were performed using IBM\textsuperscript{®} SPSS Statistics, v20.0.0.1 and v22 (SPSS Inc., Chicago, Illinois, USA). Accordance between measurements was assessed using the Bland-Altmann test. Differences between groups were examined using the Student’s t-test and the Mann-Whitney U test where appropriate and correlations of parameters were analysed using Spearman’s rank correlation with 95% confidence interval (CI 95%). P-values < 0.05 were considered statistically significant. Means were expressed plus/minus standard error of the mean (SEM) or standard deviation (SD) where appropriate.

**RESULTS**

**Baseline characteristics and Treatment outcome**
Baseline characteristics of the 86 patients included in the study are shown in Table 1. A total of 8 patients had HBsAg-loss (4 HBeAg- positive and 4 HBeAg-negative patients) and 11 of 41 HBeAg-positive patients had HBeAg-loss. CR was achieved in 14/41 HBeAg-positive and 17/45 HBeAg-negative patients. Mean log IP-10 plasma level at baseline was 3.03 log pg/mL (+/- 0.03 log pg/mL). Mean log IP-10 plasma levels at baseline were slightly higher in HBeAg-positive patients compared to HBeAg-negative patients (3.07 +/- 0.03 versus 2.99 +/- 0.04 log pg/mL), but this was not statistically significant. Mean intensity of IP-10 mRNA expression in liver tissue was 0.156 log (+/- standard deviation (SD) 1.05 log). There was no difference in baseline IP-10 levels and IP-10 mRNA expression in liver between HBeAg-positive and HBeAg-negative patients.
Table 1. Baseline characteristics of patients treated with peginterferon and adefovir for 48 weeks, according to HBeAg-status.

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>HBeAg-positive</th>
<th>HBeAg-negative</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>41</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong> (male / female) (%)</td>
<td>32 (78) / 9 (22)</td>
<td>31 (69) / 14 (31)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Age</strong> (mean, years) (SD)</td>
<td>35.2 (9.3)</td>
<td>43.1 (9.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Interferon-naive</strong> (N) (%)</td>
<td>32 (78)</td>
<td>32 (71)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HBV-DNA</strong> (mean log IU/mL) (SD)</td>
<td>8.1 (1.2)</td>
<td>5.5 (1.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>HBV Genotype</strong> (N) (%)</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>A</td>
<td>17 (42)</td>
<td>9 (20)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>8 (20)</td>
<td>7 (16)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>7 (17)</td>
<td>5 (11)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>7 (17)</td>
<td>17 (38)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2 (5)</td>
<td>7 (16)</td>
<td></td>
</tr>
<tr>
<td><strong>HBsAg</strong> (mean log IU/mL) (SD)</td>
<td>4.31 (0.75)</td>
<td>3.33 (0.69)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ALT</strong> (xULN)* (SD)</td>
<td>4.2 (5.6)</td>
<td>2.2 (1.7)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>HBsAg-loss</strong> (N) (%)</td>
<td>4 (10)</td>
<td>4 (8)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HBeAg-loss</strong> (N) (%)</td>
<td>11 (27)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Combined Response</strong> (N) (%)</td>
<td>14 (34)</td>
<td>17 (38)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>IP-10 plasma</strong> (mean log pg/mL) (SD)</td>
<td>3.07 (0.27)</td>
<td>2.99 (0.29)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Liver biopsy</strong> (N) (%)</td>
<td>18 (44)</td>
<td>22 (49)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HAI-score</strong> (median) (range)</td>
<td>5 (1-13)</td>
<td>5 (1-13)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Ishak fibrosis score</strong> (median) (range)</td>
<td>1 (0-6)</td>
<td>1 (0-6)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Cirrhosis</strong> (N) (%)</td>
<td>2 (7)</td>
<td>9 (23)</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>IP-10 mRNA expression</strong> (mean log copies vs 2 housekeeping genes) (SD)</td>
<td>0.23 (0.56)</td>
<td>0.09 (0.45)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* ULN: upper limit of normal, >45 U/L for men and >34 U/L for women

Baseline plasma IP-10 levels in HBeAg-positive and HBeAg-negative patients

Baseline IP-10 plasma levels were slightly higher in both HBeAg-positive (3.10 +/- 0.12 log pg/mL) and -negative (3.07 +/- 0.05 log pg/mL) patients with HBsAg-loss at W72, although not statistically different. In HBeAg-positive patients, plasma IP-10 levels at baseline were significantly higher in patients with CR versus NR (3.20 +/- 0.06 log pg/mL versus 3.00 +/- 0.05 log pg/mL) (p=0.03). This difference was not observed in HBeAg-negative patients (3.00 +/- 0.07 log pg/mL versus 3.00 +/- 0.06 log pg/mL), which is shown in Figure 1.

In HBeAg-positive patients, baseline plasma IP-10 levels were higher in patients with a decline in HBsAg of ≥0.5 log IU/mL at W12 compared to those without this decline (3.28
+/- 0.06 log pg/mL versus 2.98 +/- 0.05 log pg/mL) (p = 0.001). The same was observed in HBeAg-positive patients with a decline in HBsAg of ≥ 0.5 log IU/mL at W72 compared to those without this decline (3.19 +/- 0.07 log pg/mL versus 3.01 +/- 0.05 log pg/mL (p = 0.049)). These differences were not observed in HBeAg-negative patients. This is shown in Figure 2.

Baseline IP-10 plasma levels and decline in HBsAg of ≥ 0.5 log IU/mL at W12 were combined in HBeAg-positive patients to predict combined response. If baseline IP-10 levels were higher than the mean (3.07 log pg/mL) and there was a decline in HBsAg of ≥ 0.5 log IU/mL at W12, the chance to achieve CR was 63%. In patients with baseline IP-10 plasma levels higher than the mean (3.07 log pg/mL) without a decline in HBsAg of ≥ 0.5 log IU/mL at W12, the chance to achieve CR was 33%. In patients with baseline IP-10 plasma levels below the mean without a decline in HBsAg, CR was only achieved in 19%. This is shown in Table 2.

Correlation of baseline IP-10 plasma levels with different markers of response
Baseline plasma IP-10 levels were correlated with baseline ALT in both HBeAg-positive and -negative patients (both p < 0.001). In HBeAg-negative patients baseline IP-10 levels were also correlated with baseline HBV-DNA (p = 0.01), but this was not the case in HBeAg-positive patients. In HBeAg-positive, but not in HBeAg-negative patients, a correlation was seen between baseline plasma IP-10 levels and HAI-score (p = 0.025). There was no correlation seen in HBeAg-positive or -negative patients between plasma IP-10 levels at baseline and HBsAg-levels or Ishak-score (liver fibrosis) in liver biopsy specimens (Table 3).

Figure 1. Baseline IP-10 plasma levels in HBeAg-positive and -negative patients according to response to therapy. HBeAg-positive patients with CR have higher IP-10 levels at baseline as compared to patients with NR.

Figure 2. High baseline IP-10 is associated with a more pronounced decline in HBsAg at Week 12 and Week 72 in HBeAg-positive patients.
Table 2. Combination of baseline IP-10 plasma levels (≥ or < mean 3.07 log pg/mL) and decline in HBsAg-levels ≥ 0.5 log IU/mL at W12

<table>
<thead>
<tr>
<th>IP-10 BL ≥ 3.07 log</th>
<th>HBsAg W12 ≥ 0.5 log ↓</th>
<th>Combined Response</th>
<th>Total</th>
<th>Spearman’s Correlation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>7</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>–</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>14</td>
<td>26</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 3. Correlation of IP-10 levels with different baseline parameters (ALT, HAI-score, HBV DNA, HBsAg, Ishak Fibrosis score).

<table>
<thead>
<tr>
<th>IP-10 plasma baseline Baseline</th>
<th>HBeAg-positive</th>
<th>HBeAg-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman’s Rho</td>
<td>p-value</td>
</tr>
<tr>
<td>ALT</td>
<td>0.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAI-score</td>
<td>0.40</td>
<td>0.025</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>0.08</td>
<td>NS</td>
</tr>
<tr>
<td>HBsAg</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Ishak Fibrosis score</td>
<td>0.14</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figure 3. Correlation of intrahepatic IP-10 mRNA expression with plasma IP-10 levels at baseline: stronger correlation in HBeAg-positive than in HBeAg-negative CHB patients

IP-10 mRNA expression in liver tissue at baseline
In 18 of 41 HBeAg-positive patients and in 22 of 45 HBeAg-negative patients IP-10 mRNA expression in liver tissue at baseline was determined and correlation with IP-10 plasma levels at baseline was assessed. Mean expression of IP-10 mRNA in liver
was 0.156 log copies vs 2 housekeeping genes (+/- SD 1.05 log copies) in general. In HBeAg-positive patients mean expression was 2.30 log copies vs 2 housekeeping genes (+/- SD 0.56 log copies), and in HBeAg-negative patients mean mRNA expression was 1.01 log copies vs 2 housekeeping genes (+/- SD 0.45 log copies). There was a correlation between IP-10 mRNA expression in liver and plasma IP-10 levels (R=0.55, p=0.002) in the total group, as well as in both HBeAg-positive and HBeAg-negative patients separately (Figure 3). This correlation, however, was stronger in HBeAg-positive patients (R = 0.79, 95 % CI 0.51 – 0.92, p < 0.001) than in HBeAg-negative patients (R = 0.53, 95 % CI 0.12 – 0.78, p = 0.01).

Correlation of intrahepatic IP-10 mRNA expression with different markers of response

Intrahepatic IP-10 mRNA expression was correlated with baseline ALT levels and HAI score in both HBeAg-positive and –negative patients. In HBeAg-negative patients intrahepatic IP-10 mRNA expression was also correlated with baseline HBV-DNA level, but this was not the case in HBeAg-positive patients. There was no correlation between intrahepatic IP-10 mRNA expression and HBsAg-levels or Ishak-score (liver fibrosis), both in HBeAg-positive and HBeAg-negative patients (Table 4).

Plasma IP-10 levels during treatment

At D3, mean IP-10 plasma levels increased two-fold (0.3 log pg/mL) compared to baseline (from 1.95 log pg/mL to 2.15 log pg/mL). At W1 IP-10 levels decreased 0.2 log pg/mL compared to D3, to a level still 0.14 log pg/mL higher than at baseline (2.09 log pg/mL, p<0.001). These levels were not significantly different between HBeAg-positive and HBeAg-negative patients at D3 (2.21 versus 2.22 log pg/mL) or W1 (both 2.08 pg/mL). At W18 and at EOT IP-10 plasma levels declined further to a level comparable to the baseline value. No significant differences in plasma IP-10 levels in HBeAg-positive and HBeAg-negative patients were observed (Figure 4).

In HBeAg-positive patients, the increase from baseline to D3 was significantly greater in patients with NR versus patients with CR (0.35 log pg/mL versus 0.08 log pg/mL, p=0.003) (Figure 5).

Table 4. Correlation of intrahepatic IP-10 mRNA expression with different baseline parameters (ALT, HAI-score, HBV DNA, HBsAg, Ishak Fibrosis score).

<table>
<thead>
<tr>
<th>IP-10 plasma mRNA expression</th>
<th>HBeAg-positive</th>
<th>HBeAg-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Spearman’s Rho</td>
<td>p-value</td>
</tr>
<tr>
<td>ALT</td>
<td>0.48</td>
<td>0.046</td>
</tr>
<tr>
<td>HAI-score</td>
<td>0.47</td>
<td>0.051</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>-0.27</td>
<td>NS</td>
</tr>
<tr>
<td>HBsAg</td>
<td>-0.35</td>
<td>NS</td>
</tr>
<tr>
<td>Ishak Fibrosis score</td>
<td>0.19</td>
<td>NS</td>
</tr>
</tbody>
</table>
**DISCUSSION**

In this study we found that in HBeAg-positive patients, but not in HBeAg-negative patients, baseline IP-10 plasma levels were higher in patients with combined response (HBeAg-loss, ALT-normalization and HBV-DNA < 2,000 IU/mL) compared to those with non-response. These findings are in concordance with earlier studies.\(^{32,41}\) We saw no significant relation between IP-10 levels in plasma and HBsAg-loss. Earlier studies showed contradicting results. Some studies demonstrated higher baseline IP-10 levels in patients with HBsAg-loss,\(^{33,42}\) whereas others did not, which may be explained by the small proportion of patients who achieved HBsAg-loss.\(^{32,41,43}\) We did observe, however, higher baseline IP-10 levels in HBeAg-positive patients who had a decline of HBsAg-level of more than 0.5 log IU/mL at Week 12. This is in agreement with earlier studies showing that high baseline IP-10 plasma levels are associated with HBsAg-decline in both HBeAg-positive and -negative patients treated with interferon-based therapy\(^ {32,41}\) or with NA.\(^ {33,42-44}\) Our results show that baseline IP-10 level in plasma was valuable in predicting response to (finite) peginterferon-based therapy in HBeAg-positive CHB patients, especially when used in conjunction with decline of HBsAg level at Week 12.
during treatment. Combining these two markers may help to identify patients with high and low chance of achieving a combined response. To translate this into clinical practice, early cessation (after Week 12) of peginterferon-based treatment may be considered in those patients with a low likelihood of achieving combined response, being those with low baseline IP-10 levels (< 3 log pg/mL) and no decline in HBsAg at Week 12.

As the combination of peginterferon and adefovir is not a widely used treatment modality, we believe that our findings may be applicable to finite peginterferon-based treatment options such as the recently described combination of peginterferon and tenofovir or peginterferon monotherapy.

We observed a clear correlation between IP-10 in plasma and IP-10 mRNA expression in the liver in CHB patients, which was earlier described in CHB and in chronic hepatitis C (CHC) patients. This suggests that plasma IP-10 is a reflection of intrahepatic immune activity in both in CHB and CHC patients. The fact that IP-10 mRNA expression in the liver was also associated with ALT-levels supports this hypothesis. Given the function of IP-10 being a chemo-attractant for inflammatory cells, high intrahepatic IP-10 expression is essential for migration of leukocytes into the liver in response to HBV infection. Another inflammatory cytokine, CXCL-9, related to IP-10 was also important for the immune response against HBV and may predict treatment outcome. High baseline expression of the chemokine CXCL9, which shares its receptor (CXCL3) with IP-10, was associated with response to interferon-based therapy in both HBeAg-positive and -negative CHB patients. Furthermore, the ligand programmed death-1 (PD-1), expressed on activated CD8+ and CD4+ T-cells, has been associated with T-cell exhaustion. A relation between PD-1, CXCL9 and expression of IP-10 (intrahepatic and peripheral) is mechanistically explained by the fact that IP-10 and CXCL9 are encoded by an interferon-stimulated gene (ISG) which is induced by type I interferon and activates T-lymphocytes.

The relation between IP-10 (in plasma and liver) and ALT level or HAI score we observed in HBeAg-positive patients as well as between IP-10 (in plasma and liver) and HBV-DNA in HBeAg-negative patients was shown in earlier studies. This supports our suggestion that IP-10 is a reflection of intrahepatic immune activation. In addition, pre-existent immune activation is important for virological response in HBeAg-positive patients, whereas in HBeAg-negative patients, HBV-DNA level is a more important response marker.

Our study is the first study to describe IP-10 kinetics early after start of interferon-based treatment, showing a clear increase in plasma IP-10 levels at D3 after start of treatment. Subsequently, at W1 IP-10 levels declined to a level still higher than baseline, followed by a level comparable to baseline at W18 and at EOT. This is in agreement with other studies, which showed a decline in plasma IP-10 levels during IFN-based therapy at W12 and W24 compared to baseline. The two-to threefold increase in IP-10 plasma levels shortly after start of therapy in our study was also described in patients with CHC. In CHC patients a dose-dependent two- to nine fold rise in plasma IP-10 levels was seen at day 1-3 after start of interferon-based therapy. The fact that in all patients in our study at D3 a significant rise in plasma IP-10 was observed indicates that early after start of interferon-based treatment, the immune activation of responders and non-responders was similar. In CHC patients treated with high-dose interferon a similar increase of IP-10 level was observed. This suggests that the increase in IP-10
levels early after start of (peg)interferon-based treatment is the result of a non-specific stimulation of the Interferon type I immune response, causing the induction of multiple ISG’s, which is not specific for an immune response provoking response to therapy.

Our study population consists of HBeAg-positive and HBeAg-negative patients, and as a consequence the numbers of patients in the different groups remain relatively small. As a result, we might have missed possible relationships between serum IP-10 or intrahepatic IP-10 mRNA and treatment response parameters in HBeAg-negative patients. However, the fact that we did find a statistically significant relation between IP-10 and treatment response in HBeAg-positive patients of comparable size, suggests that there is a difference between HBeAg-positive and HBeAg-negative CHB patients. This is also in line with earlier study results of serum IP-10 levels in HBeAg-positive and- negative CHB patients.32

In conclusion, we found that plasma IP-10 levels and IP-10 mRNA expression in the liver at baseline were correlated with each other, especially in HBeAg-positive patients. Higher IP-10 levels in plasma seem to be associated with combined response in HBeAg-positive, but not in HBeAg-negative CHB patients treated with peginterferon-based therapy. Our findings underline the importance of pre-treatment immune activation in HBeAg-positive CHB patients as a predictor of response to antiviral immune modulating therapy, of which plasma IP-10 levels and intrahepatic IP-10 mRNA expression are a reflection.

ACKNOWLEDGEMENTS AND DISCLOSURES

Acknowledgements
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Disclosures
This study was financially funded by Gilead Sciences with a non-restricted grant. The sponsor has had no involvement in the design of the study, the collection, analysis and interpretation of data, in writing the report, and in submitting the data for publication.
REFERENCES


Clinical Studies on Hepatitis B, C, and E Virus Infection

CHAPTER 3

Peg-interferon plus Nucleotide Analogue Treatment versus no Treatment in Patients with Chronic Hepatitis B with a Low Viral Load: a Randomized Controlled, Open Label Trial

* contributed equally

ABSTRACT

Background
Antiviral treatment is currently not recommended for patients with chronic hepatitis B with a low viral load. However, they might benefit from acquiring a functional cure (hepatitis B surface antigen [HBsAg] loss with or without formation of antibodies against hepatitis B surface antigen [anti-HBs]). We assessed HBsAg loss during peg-interferon-alfa-2a (peg-IFN) and nucleotide analogue combination therapy in patients with chronic hepatitis B with a low viral load.

Methods
In this randomised controlled, open-label trial, patients were enrolled from the Academic Medical Center (AMC), Amsterdam, Netherlands. Eligible patients were HBsAg positive and hepatitis B e antigen (HBeAg) negative for more than 6 months, could be treatment naive or treatment experienced, and had alanine aminotransferase (ALT) concentrations less than 5 × upper limit of normal (ULN). Participants were randomly assigned (1:1:1) by a computerised randomisation programme (ALEA Randomisation Service) to receive peg-IFN 180 µg/week plus adefovir 10 mg/day, peg-IFN 180 µg/week plus tenofovir disoproxil fumarate 245 mg/day, or no treatment for 48 weeks. The primary endpoint was the proportion of patients with serum HBsAg loss among those who received at least one dose of study drug or had at least one study visit (modified intention-to-treat population [mITT}). All patients have finished the initial study of 72 weeks and will be observed for up to 5 years of follow-up. This study is registered with ClinicalTrials.gov, number NCT00973219.

Findings
Between Aug 4, 2009, and Oct 17, 2013, 167 patients were screened for enrolment, of whom 151 were randomly assigned (52 to peg-IFN plus adefovir, 51 to peg-IFN plus tenofovir, and 48 to no treatment). 46 participants in the peg-IFN plus adefovir group, 45 in the peg-IFN plus tenofovir group, and 43 in the no treatment group began treatment or observation and were included in the mITT population. At week 72, two (4 %) patients in the peg-IFN plus adefovir group and two (4 %) patients in the peg-IFN plus tenofovir group had achieved HBsAg loss, compared with none of the patients in the no treatment group (p = 0·377). The most frequent adverse events (> 30%) were fatigue, headache, fever, and myalgia, which were attributed to peg-IFN dosing. Two (4 %) serious adverse events were reported in the peg-IFN plus adefovir group (admission to hospital for alcohol-related pancreatitis [week 6; n = 1] and pregnancy, which was electively aborted [week 9; n = 1]), three (7 %) in the peg-IFN plus tenofovir group (admission to hospital after a suicide attempt during a severe depression [week 23; n = 1], admission to hospital for abdominal pain [week 2; n = 1], and an elective laminectomy [week 40; n = 1]), and three (7 %) in the no treatment group (admission to hospital for septic arthritis [week 72; n = 1], endocarditis [week 5; n = 1], and hyperthyroidism [week 20; n = 1]).

Interpretation
In patients with chronic hepatitis B with a low viral load, combination treatment (peg-IFN plus adefovir and peg-IFN plus tenofovir) did not result in significant HBsAg loss compared with no treatment, which does not support the use of combination treatment in this population of patients.
INTRODUCTION

Worldwide, there are over 350 million people chronically infected with the hepatitis B virus (HBV), with the highest prevalence in South-East Asia and Africa. Every year HBV infection is responsible for over 780,000 deaths. In acute infection an adequate immune response and resolution of the virus with lifelong protective immunity is seen in approximately 95% of adult people. Perinatal transmission of HBV from mother to neonate has a higher chance to result in chronic infection. When the host immune response is inadequate and the infection persists, patients become chronically infected and are at risk to develop liver cirrhosis and hepatocellular carcinoma. The mechanism of developing chronic hepatitis B virus (HBV) infection, rather than clearing acute infection, is not fully understood. It has been associated with impairment of the innate and adaptive immune responses. In most individuals with acute HBV infection that spontaneously resolve, strong and broad virus specific T-cell responses directed against the HBV-infected hepatocytes can be detected. However, these responses are weak and narrowly focused in patients who develop chronic HBV infection, resulting in low levels of antiviral cytokines and attenuated cytotoxic T-lymphocyte (CTL) activity. During chronic HBV infection inflammatory activity and HBV load may fluctuate in some patients whereas in others inflammation is absent and HBV load remains low.

Many cytokines and chemokines have been identified to be involved in immune reactions in response to HBV infection. Interferon-γ-inducible protein-10 kDa (IP-10/CXCL10) is a non-EXR-CXC chemokine and is a chemo-attractant for T-lymphocytes, monocytes, and NK-cells. This cytokine and especially its relation to viral clearance and response to antiviral therapy have extensively been described in acute and chronic hepatitis C virus (HCV) infection. Next to this, it was shown in chronic HCV patients that intrahepatic IP-10 mRNA expression and plasma IP-10 levels are correlated with each other. In chronic HBV patients, serum IP-10 seems to be higher than in healthy controls. Serum IP-10 is positively correlated with HBV-DNA, ALT levels and progressive disease in chronic HBV infection. Next to this, high pre-treatment IP-10 levels have been associated with HBeAg-loss during or after treatment with peginterferon or nucleo(s)tid analogue(s) (NAs). Combination therapy with peginterferon and adefovir or tenofovir in active CHB patients showed higher response rates than monotherapy with either medicament alone (peginterferon or NAs).

It is not entirely clear whether IP-10 levels at baseline or changes in IP-10 levels during treatment predict response to treatment in active CHB patients, treated with a combination of peginterferon and a nucleo(s)tid analogue (NA). Aside from this, a correlation between intrahepatic IP-10 mRNA expression and plasma IP-10 levels in chronic HBV infection has not been investigated. Our aim was to establish if IP-10 levels at baseline and early during therapy can predict treatment outcome, and to establish if IP-10 mRNA expression in liver tissue is correlated with plasma IP-10 levels in CHB patients.
METHODS

Study design and participants
This investigator-initiated, prospective, open-label, randomised controlled trial was done at the Academic Medical Center (AMC), Amsterdam, Netherlands.

Patients with chronic hepatitis B aged 18–70 years with a low viral load were enrolled after assessment of eligibility. Hepatitis B virus DNA below a threshold of 20,000 IU/mL was chosen as low viral load according to local and international guidelines available at the time of study initiation. Inclusion criteria were documented HBsAg positivity and HBeAg negativity for more than 6 months. Exclusion criteria were concurrent infection with hepatitis C virus, hepatitis delta virus, or HIV; decompensated liver disease, hepatocellular carcinoma, or a history of bleeding from oesophageal varices; pregnancy or breastfeeding; and alanine aminotransferase (ALT) levels greater than five times the upper limit of normal (5 × ULN). Patients were either treatment naive, or had received peg-IFN or nucleos(t)ide analogues more than 6 months before inclusion. The full eligibility criteria and screening assessments are provided in the appendix (p 1–3).

The study complied with the Declaration of Helsinki and the principles of Good Clinical Practice and was approved by a legally instituted ethical committee. All patients gave written informed consent.

Randomisation and masking
Patients were randomly assigned (1:1:1) to receive pegylated interferon alfa-2a (Pegasys; F Hoffman-La Roche, Basel, Switzerland) 180 µg/week, subcutaneously in combination with adefovir dipivoxil (Hepsera; Gilead Sciences, Foster City, CA, USA) 10 mg orally once daily for 48 weeks; peg-interferon alfa plus tenofovir disoproxil fumarate (Viread; Gilead Sciences) 245 mg orally once daily for 48 weeks; or no treatment (current standard of care) for 48 weeks. Patients were enrolled by the subinvestigators and randomly assigned by a computerised randomisation programme (ALEA Randomisation Service). Randomisation was stratified according to hepatitis B virus genotype A, non-A (genotype B–G), or indeterminable genotype. Patients and investigators were not masked. After 48 weeks, treatment was discontinued and all patients were followed up until week 72.

Procedures
Routine examinations and laboratory tests were done with 2 weekly intervals for the first 2 months and 6 weekly intervals thereafter (appendix pp 4–7). The viral load was assessed before selecting or approaching eligible patients, if available from the medical chart. Furthermore, viral load was assessed at the time of screening and at day 1 of treatment, of which the latter was used as baseline hepatitis B virus DNA level. Due to subsequent changes in expert opinion on the classification of low viral load, a subgroup analysis on patients with hepatitis B virus DNA level less than 2000 IU/mL (often referred to as inactive carriers) was done.

Plasma hepatitis B virus DNA level was determined by the COBAS TaqMan assay (F Hoffmann-La Roche, Basel, Switzerland). Serum HBsAg level was quantified by using the Abbott HBsAg Architect assay (Abbott Diagnostics, Abbott Park, IL, USA). Qualitative detection of serum HBsAg, antibody to HBsAg (anti-HBs), HBeAg, and antibody to HBeAg (anti-HBe) was done by an enzyme immunoassay (AxSYM; Abbott Laboratories,
Abbott Park, IL, USA). ALT levels were expressed as absolute values (U/L) or relative to the ULN range. ALT reference values were 45 U/L for men and 34 U/L for women. For histological assessment of liver biopsies the modified Ishak scoring system was applied, based on a 0–18 score for necroinflammation and a 0–6 score for fibrosis.33

Outcomes
The primary objective was the proportion of patients with HBsAg loss. HBsAg loss was defined as undetectable serum HBsAg by AxSYM (<0·05 IU/mL). The secondary objectives were the proportion of patients with HBsAg loss who also had anti-HBs seroconversion (defined as anti-HBsAg > 10 IU/L), and the identification of predictive markers of treatment response. Post-hoc analyses assessed HBsAg loss at week 48 and HBsAg decline at weeks 48 and 72. Patients were considered to be non-responders when not meeting the criteria for HBsAg loss or HBsAg decline. Adverse events were assessed at each study visit through anamnesis and laboratory testing.

Statistical analysis
The sample size calculation was based on the primary endpoint (HBsAg loss at week 72). The assumed response rates were 20 % for patients treated in each combination group versus 1 % for patients in the control group (receiving no treatment).25 A group sample of 44 patients in the control group was determined to detect a difference with either of the treatment arms at the α level of 0·05 (two-sided Fisher’s exact test) with a power of 81 %. Assuming a 10 % drop-out rate, 50 patients or more were needed in each group.

In the primary analysis, differences in the proportion of patients with HBsAg loss in each group were compared with a Pearson χ² test. In this analysis, a modified intention-to-treat (mITT) model was applied, including all patients who received at least one dose of study drugs (treatment groups) or had at least one study visit (no treatment group). Patients who prematurely discontinued treatment were scored as non-responders. Patients with missing data were considered non-responders. In the secondary analysis on HBsAg decline, only patients who completed 48 weeks of treatment and 24 weeks of treatment-free follow-up were included (per-protocol model). Use of the per-protocol population for the secondary analysis allowed avoidance of a high proportion of missing data from patients who discontinued.

Baseline and on-treatment variables were compared between study groups using Welch’s t, Students t, Mann-Whitney U, Kruskall-Wallis, Pearson’s χ², or Fisher’s exact tests. The associations between variables as potential predictors of HBsAg loss or HBsAg decline were examined by multivariable logistic regression analysis. Data were analysed with IBM SPSS Statistics, version 21. All p values are two sided and values below 0·05 were considered statistically significant.

This study is registered with ClinicalTrials.gov, number NCT00973219.

Role of the funding source
The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.
RESULTS

Between Aug 4, 2009, and Oct 17, 2013, 167 patients were screened for participation in the study, of whom 151 patients were randomly assigned (nine did not meet inclusion criteria and seven withdrew before randomisation).

52 Participants were assigned to peg-IFN plus adefovir, 51 were assigned to peg-IFN plus tenofovir, and 48 were assigned to no treatment (Figure 1). 17 patients did not start allocated intervention (reasons stated in Figure 1). Between Sept 7, 2009, and Dec 12, 2013, 134 patients started intervention or no treatment and were included in the mITT population: 46 of 52 in the peg-IFN plus adefovir group, 45 of 51 in the peg-IFN plus tenofovir group, and 43 of 48 in the no treatment group. Of 134 patients, 12 prematurely discontinued the study: five in the peg-IFN plus adefovir group, six in the peg-IFN plus tenofovir group, and one in the no treatment group (Figure 1).

Reasons for treatment discontinuation in the peg-IFN plus adefovir group were alcohol-related pancreatitis (n = 1; at 6 weeks), dizziness (n = 1; at 8 weeks), hair loss (n = 1; at 24 weeks), back pain (n = 1; at 38 weeks), and laboratory abnormalities with concomitant alcohol abuse (n = 1; at 42 weeks). Treatment in the peg-IFN plus tenofovir group was discontinued because of general interferon-related side-effects (n=2; at 1 and 8 weeks), nausea and vomiting (n = 1; at 12 weeks), depression (n = 2; at 12 and 23 weeks), and hair loss (n = 1; at 24 weeks). One patient in the no treatment group was lost to follow-up after 6 weeks. Thus, the per-protocol population consisted of 41 patients in the peg-IFN plus adefovir group, 39 in the peg-IFN plus tenofovir group, and 42 in the no treatment group.

Patient characteristics are shown in Table 1. Patients were predominantly men (75 [56 %] of 134), with a mean age of 43 years (SD 11). Ethnic background was mixed, as were hepatitis B virus genotypes. Most patients were interferon naive, and none of the patients had received a nucleos(t)ide analogue within 6 months of entering the trial. All patients were HBeAg negative, with a low viral load (mean hepatitis B virus DNA 274 log_{10} IU/mL [SD 1-10]), normal ALT levels (median 27 U/L [IQR 21–37]), and minimal liver fibrosis (mean Fibroscan value 5·4 kPa [SD 1·9]). The modified hepatic activity index (HAI) was low (median 2 [IQR 2–3]) and none of the patients had evidence of liver cirrhosis; the maximum Ishak’s fibrosis score was 3 (or a maximum Fibroscan value of 8-1 kPa in patients with no liver biopsy available).

19 Patients required peg-IFN dose reductions (11 in the peg-IFN plus adefovir group and eight in the peg-IFN plus tenofovir group).

At Week 72, two (4%) patients in the peg-IFN plus adefovir group, two (4%) patients in the peg-IFN plus tenofovir group, and no patients in the no treatment group had HBsAg loss (p = 0.377). Of the four patients who were HBsAg negative at week 72, one patient was not peg-IFN naive. Patients with HBsAg loss had hepatitis B virus genotype A (one patient in the peg-IFN plus adefovir group), genotype B (two patients in the peg-IFN plus tenofovir group), or indeterminable (one patient in the peg-IFN plus adefovir group). Three of four patients had anti-HBs higher than 10 IU/L (n = 1 from peg-IFN plus adefovir group and n = 2 from peg-IFN plus tenofovir group). HBsAg response rates at Week 48 and Week 72 are shown in Table 2.

At Week 48, one patient (2%) in the peg-IFN plus adefovir group, three patients (7%) in the peg-IFN plus tenofovir group, and no patients in the no treatment group had HBsAg loss (p = 0.171). Of four patients with HBsAg loss at Week 48, three had anti-HBs higher
than 10 IU/L. During follow-up, the patient without anti-HBs conversion (in the peg-IFN plus tenofovir group, indeterminable genotype) seroreverted to HBsAg positivity to levels around the detection limit at Week 72. The course of virological and biochemical parameters per patient who achieved HBsAg loss during treatment, or follow-up, or both (n = 5), is shown in the appendix (p 8).

In a post-hoc per-protocol analysis, mean HBsAg level had declined significantly in all study groups at Week 48 compared with at baseline: mean –0.61 log_{10} IU/mL (SD 0.92) reduction for the peg-IFN plus adefovir group (p = 0.0001), –0.61 log_{10} IU/mL (0.96) reduction for the peg-IFN plus tenofovir group (p = 0.0003), and –0.06 log_{10} IU/mL (0.18) reduction for the no treatment group (p = 0.042; Table 3). No difference in HBsAg

Figure 1. Trial profile

*Patients who did not receive allocated intervention; peg-IFN plus adefovir: lost to follow-up (n = 3), patient withdrew consent (n = 2), worsening of psychiatric symptoms (n = 1); peg-IFN plus tenofovir: lost to follow-up (n = 2), patient withdrew consent (n = 1), worsening of psychiatric symptoms (n = 1), initiation of lamivudine (n = 1), no health insurance (n = 1); no treatment: lost to follow-up (n = 2), moved abroad (n = 2) and patient withdrew consent (n = 1). † Reasons for treatment discontinuation are specified in the Results section.
Table 1. Baseline characteristics of the modified intention-to-treat population (n=134)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Treatment arms</th>
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<tr>
<td></td>
<td>Arm I; Peg-IFN + adefovir n = 46</td>
<td>Arm II; Peg-IFN + tenofovir n = 45</td>
<td>Arm III; No Treatment n = 43</td>
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<tr>
<td>Women, n (%)</td>
<td>18 (39)</td>
<td>24 (53)</td>
<td>17 (40)</td>
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<td>Men, n (%)</td>
<td>28 (61)</td>
<td>21 (47)</td>
<td>26 (60)</td>
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<td>Age, years, mean (sd)</td>
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<td>43 (12)</td>
<td>41 (10)</td>
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<td>Ethnicity</td>
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<td>Caucasian, n (%)</td>
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<td>Asian, n (%)</td>
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<td>11 (24)</td>
<td>7 (16)</td>
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<td>African, n (%)</td>
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<td>11 (24)</td>
<td>14 (33)</td>
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<td>South American, n (%)</td>
<td>8 (17)</td>
<td>9 (20)</td>
<td>5 (12)</td>
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<td>IFN naïve, n (%)</td>
<td>43 (93)</td>
<td>42 (93)</td>
<td>43 (100)</td>
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<tr>
<td>ALT, U/L, median (iqr)</td>
<td>27 (21–42)</td>
<td>25 (19–30)</td>
<td>30 (21–47)</td>
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<tr>
<td>HBV Genotype</td>
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<td>Indeterminable, n (%)</td>
<td>9 (20)</td>
<td>11 (24)</td>
<td>8 (19)</td>
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<td>G, n (%)</td>
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<td>HBV-DNA, log₁₀ IU/mL, mean (sd)</td>
<td>2.65 (1.23)</td>
<td>2.79 (1.03)</td>
<td>2.79 (1.04)</td>
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<tr>
<td>HBV-DNA &lt;2,000 IU/mL, n (%)</td>
<td>33 (72)</td>
<td>28 (62)</td>
<td>30 (70)</td>
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<tr>
<td>HBsAg, log10 IU/mL, mean (sd)</td>
<td>3.21 (0.98)</td>
<td>3.31 (0.76)</td>
<td>3.06 (0.88)</td>
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<td>Fibroscans performed, n (%)</td>
<td>41 (89)</td>
<td>36 (80)</td>
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<tr>
<td>Vvalue (kPa) mean (sd)</td>
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<td>5.4 (1.8)</td>
<td>5.8 (2.0)</td>
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<td>Liver biopsy</td>
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<td>Liver biopsies performed, n (%)</td>
<td>40 (87)</td>
<td>39 (87)</td>
<td>19 (44)</td>
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<tr>
<td>Mean biopsy length, mm, median (iqr)</td>
<td>16 (12–22)</td>
<td>20 (15–26)</td>
<td>13 (11–15)</td>
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<tr>
<td>Mean portal fields, n, median (iqr)</td>
<td>11 (7–14)</td>
<td>15 (8–18)</td>
<td>9 (7–14)</td>
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<td>Median inflammatory score, median (iqr)</td>
<td>2 (2–3)</td>
<td>2 (1–3)</td>
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<td>Median Ishak fibrosis score, median (iqr)</td>
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<td>1 (1–1)</td>
<td>1 (1–1)</td>
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<td>Median steatosis, grade, median (iqr)</td>
<td>0 (0–1)</td>
<td>0 (0–1)</td>
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<tr>
<td>Median % HBsAg staining, median (iqr)</td>
<td>10 (1–35)</td>
<td>25 (5–50)</td>
<td>10 (1–25)</td>
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decline was noted between the two treatment groups (p=0.990). However, HBsAg declined more strongly in the peg-IFN plus adefovir group (p=0.0005) and the peg-IFN plus tenofovir group (p=0.001) than in the no treatment group (Figure 2A, B). An HBsAg decline of more than 1·0 log10 IU/mL was noted in 17 (21%) treated patients (nine [22%] in the peg-IFN plus adefovir group and eight [21%] in the peg-IFN plus tenofovir group), but in none of the no treatment patients (p = 0·001).

During follow-up, HBsAg levels remained significantly lower than pretreatment levels in all groups: mean reduction –0·53 log10 IU/mL (SD 0·77) at Week 72 for the peg-IFN plus adefovir group (p<0·0001), mean reduction –0·59 (0·85) log10 IU/mL reduction
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at Week 72 for the peg-IFN plus tenofovir group (p = 0·0001), and mean reduction –0·15 (0·22) log$_{10}$ IU/mL reduction at Week 72 for the no treatment group (p < 0·0001; Table 3; Figure 2A, B). Despite the slight increase in mean HBsAg levels during treatment-free follow-up of treated patients, the decline in HBsAg remained significantly larger in the peg-IFN plus adefovir group (p = 0·004) and peg-IFN plus tenofovir group (p = 0·004) than in the no treatment group.

The increase in HBsAg level during the treatment-free follow-up (HBsAg rebound) was particularly pronounced in patients with an HBsAg decline of more than 1·0 log$_{10}$ IU/mL at week 48, and 13 patients in this group remained HBsAg-positive at this timepoint (Figure 2C). Of these, 12 (92 %) of 13 had an HBsAg rebound during the treatment-free follow-up (mean increase 0·84 log$_{10}$ IU/mL at week 72 compared with week 48). The decline in hepatitis B virus DNA level and subsequent hepatitis B virus DNA rebound did not differ between patients with or without more than 1·0 log$_{10}$ IU/mL HBsAg decline (Figure 2D).

Baseline and early on-treatment variables were compared between treated patients with more than 1·0 log$_{10}$ IU/mL reduction in HBsAg level at week 48 and those with less than 1·0 log$_{10}$ IU/mL reduction (appendix p 9). Because the decline in HBsAg between the peg-IFN plus adefovir group and peg-IFN plus tenofovir group were similar, these were combined for this analysis. Baseline HBsAg levels were not significantly lower in treated patients with a strong HBsAg decline. However, significant predictors of HBsAg decline in univariable analysis were male sex (p = 0·041), higher maximum on-treatment ALT level (p = 0·003), and lower week 12 HBsAg level (p = 0·002). Both on-treatment ALT increase

| Table 3. Multivariable analysis of predictors for on-treatment HBsAg decline |
|---|---|---|---|---|---|---|
| | Univariable Analysis | Multivariable Analysis |
| | Model 1 | Model 2 |
| | B | SE | p | B | SE | p | B | SE | p |
| Female sex | -1·27 | (0·63) | 0·041 | - | - | - | - | - | - |
| HBV genotype A | -0·29 | (0·71) | 0·683 | - | - | - | - | - | - |
| Baseline ALT (log$_{10}$ U/L) | -0·02 | (0·02) | 0·397 | - | - | - | - | - | - |
| Baseline HBV-DNA (log$_{10}$ U/L) | -0·23 | (0·24) | 0·341 | - | - | - | - | - | - |
| Baseline HBsAg (log$_{10}$ U/L) | -0·40 | (0·28) | 0·160 | - | - | - | - | - | - |
| Maximum ALT (log$_{10}$ U/L) | 3·30 | (1·12) | 0·003 | - | - | - | 5·02 | (1·31) | 0·0001 |
| Max- fold-change ALT | 0·38 | (0·14) | 0·006 | 0·49 | (0·16) | 0·002 | - | - | - |
| Week 12 HBV-DNA (log$_{10}$ U/L) | -0·44 | (0·52) | 0·401 | - | - | - | - | - | - |
| Week 12 HBsAg (log$_{10}$ U/L) | -0·83 | (0·27) | 0·002 | - | - | - | -1·03 | (0·31) | 0·001 |
| Week 12 HBsAg decline | -3·80 | (1·12) | 0·001 | -3·91 | (1·20) | 0·001 | - | - | - |

Baseline and early on-treatment variables significantly associated with >1 log HBsAg reduction at week 48 in univariable logistic regression analysis were assessed in two multivariable models. HBV=hepatitis B virus. ALT=alanine aminotransferase. HBsAg=hepatitis B surface antigen. B=regression coefficient.
and HBsAg level at Week 12 were independent predictors of HBsAg decline at Week 48, in different multivariable logistic regression models (Table 3). Most patients had a baseline hepatitis B virus DNA level less than 2000 IU/mL (33 [72%] patients in the peg-IFN plus adefovir group, 28 [62%] in the peg-IFN plus tenofovir group, and 30 [70%] in the no treatment group; Table 1), including all four patients with HBsAg loss at Week 72. In this subgroup analysis, HBsAg declined more on average in the peg-IFN plus adefovir group (p = 0.010) and peg-IFN plus tenofovir group (p = 0.003) than in the no treatment group at Week 72 (appendix p 10).

The most frequent adverse events (> 30%) were fatigue, headache, fever, and myalgia, which were attributed to peg-IFN dosing (Table 4). Two (4%) serious adverse events were reported in the peg-IFN plus adefovir group (admission to hospital for alcohol-related...
### Table 4. Overview of adverse events in the modified intention-to-treat population

<table>
<thead>
<tr>
<th></th>
<th>Peg-IFN+ADV n=46</th>
<th>Peg-IFN+TDF n=45</th>
<th>No treatment n=43</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serious adverse events</strong>*</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>General</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>23</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>62%</td>
<td>5%</td>
</tr>
<tr>
<td>Headache</td>
<td>18</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>39%</td>
<td>42%</td>
<td>5%</td>
</tr>
<tr>
<td>Fever</td>
<td>16</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>35%</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>Myalgia</td>
<td>15</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>33%</td>
<td>36%</td>
<td></td>
</tr>
<tr>
<td>Other flu-like symptoms</td>
<td>9</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>20%</td>
<td>5%</td>
</tr>
<tr>
<td>Dizziness</td>
<td>8</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>17%</td>
<td>16%</td>
<td>12%</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7%</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>Back pain</td>
<td>2</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>16%</td>
<td>7%</td>
</tr>
<tr>
<td>Cough</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>7%</td>
<td></td>
</tr>
<tr>
<td>Change in menstrual pattern</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>9%</td>
<td>2%</td>
</tr>
<tr>
<td><strong>Digestive tract</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>14</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>22%</td>
<td>16%</td>
</tr>
<tr>
<td>Change in stool consistency</td>
<td>10</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>22%</td>
<td>13%</td>
<td>2%</td>
</tr>
<tr>
<td>Nausea</td>
<td>17</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>37%</td>
<td>7%</td>
<td>2%</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>7</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>29%</td>
<td></td>
</tr>
<tr>
<td>Dysgeusia</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>7%</td>
<td>2%</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td><strong>Dermatological</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin rash</td>
<td>7</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>20%</td>
<td>2%</td>
</tr>
<tr>
<td>Pruritus</td>
<td>9</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td>Alopecia</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>17%</td>
<td>7%</td>
<td></td>
</tr>
<tr>
<td>Dry mucous membranes</td>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7%</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td>Dry skin</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>9%</td>
<td></td>
</tr>
<tr>
<td><strong>Psychiatric</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression, including mood changes</td>
<td>10</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>22%</td>
<td>27%</td>
<td>2%</td>
</tr>
<tr>
<td>Concentration problems</td>
<td>2</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>Insomnia</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11%</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td><strong>Laboratory</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anemia (&lt; 6.0 mmol/L)</td>
<td>4</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td>Neutropenia (&lt;0.75x10^9 cells/L)</td>
<td>15</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>33%</td>
<td>16%</td>
<td>2%</td>
</tr>
<tr>
<td>Thrombocytopenia (&lt;75x10^9 cells/L)</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Thyroid abnormalities</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td>On-treatment ALT elevation**</td>
<td>24</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>52%</td>
<td>53%</td>
<td>7%</td>
</tr>
<tr>
<td>Off-treatment ALT elevation**</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9%</td>
<td>7%</td>
<td>9%</td>
</tr>
</tbody>
</table>
pancreatitis [week 6; n = 1] and pregnancy, which was electively aborted [week 9; n = 1]),
three (7%) in the peg-IFN plus tenofovir group (admission to hospital after a suicide attempt during a severe depression [week 23; n = 1], admission to hospital for abdominal pain [week 2; n = 1], and an elective laminectomy [week 40; n = 1]), and three (7%) in the no treatment group (admission to hospital for septic arthritis [week 72], endocarditis [week 5], and hyperthyroidism [week 20]). During treatment, 48 (53%) of 91 patients in the intervention groups (24 [52%] of 46 in the peg-IFN plus adefovir group and 24 [53%] of 45 in the peg-IFN plus tenofovir group) had ALT levels greater than 2 × ULN compared with three (7%) of 43 patients in the no treatment group (p < 0.0001). During follow-up, the rate of ALT levels greater than 2 × ULN was similar in the intervention groups (four [9%] of 46 in the peg-IFN plus adefovir group and three [7%] of 45 in the peg-IFN plus tenofovir group) versus the no treatment group (four [9%] of 43; p = 0.751).

**DISCUSSION**

Our results show no differences in functional cure, defined as HBsAg loss with or without formation of antibodies against anti-HBs between the treated and untreated patient groups. Our study is the first, to our knowledge, to analyse the effect of antiviral treatment in patients with chronic hepatitis B with a low viral load. The rationale for the study was based on the hypothesis that combining peg-IFN with a nucleos(t)ide analogue results in increased rates of HBsAg loss and anti-HBs seroconversion. Due to the relatively high rate of HBsAg loss in our cohort of patients with chronic hepatitis B with a high viral load treated with peg-IFN and adefovir, particularly in HBeAg-negative patients with low HBsAg levels, 25 we explored the effect of this combination. In our previous study in patients with a high viral load, HBsAg loss was associated with low baseline HBsAg levels. Because HBeAg-negative patients with a low viral load generally have low HBsAg levels, we hypothesised that this patient group could benefit from combination treatment. Furthermore, we hypothesised that immuno-modulation might be effective in patients with a low viral load, because they have residual hepatitis B virus-specific T-cell activity, possibly sensitive to a boost with immuno-modulatory agents. However, despite the low baseline HBsAg levels 26 and the significant residual T-cell function in this group, 29,30 combination therapy did not lead to the anticipated increased levels of functional cure. Because the definition of patients with a low viral load (inactive carriers) changed over time, a subanalysis was done, taking into account only those patients with hepatitis B virus DNA less than 2,000 IU/mL. Although all four patients with HBsAg loss were included in the
group of patients with hepatitis B with a virus viral load less than 2000 IU/mL, no statistical difference was noted between treated and untreated patients with hepatitis B with virus DNA levels less than 2000 IU/mL.

When quantitative HBsAg level was taken into account, the decline in HBsAg was significantly greater in the treatment groups than in the no treatment group. This is in line with the findings of Bourlière and colleagues\textsuperscript{28} who reported a significant decline in HBsAg levels in nucleos(t)ide analogue suppressed patients treated with peg-IFN add-on compared with no peg-IFN add-on, while no difference in functional cure was observed between the groups. Although the exact mechanism might differ, boosting the immune system in patients with a low viral load and residual T-cell function does not seem to be effective in acquiring functional cure in either of these patient groups. Furthermore, Bourlière and colleagues\textsuperscript{28} showed no increase in the proportion of patients with HBsAg loss during long-term follow-up. Whether a strong HBsAg decline can lead to future HBsAg loss in patients with chronic hepatitis B with a low viral load will be determined in our subsequent 5-year follow-up study. Here, the outcome of HBsAg decline was associated with an on-treatment ALT increase and with low HBsAg level at Week 12, indicating that the observed decline occurred early. Association of HBsAg loss and ALT flare has previously been observed in patients treated with combination therapy.\textsuperscript{24} However, because of low numbers and a rebound in HBsAg levels at Week 72 in a proportion of patients, these results should be interpreted with caution. Furthermore, this study was done in a heterogeneous population. Although this accurately reflects the mixed European population, associations with ethnicity or hepatitis B virus genotype could have been underestimated.

Peg-IFN treatment is known to be associated with severe side-effects and safety issues. In our study, despite initial consent, 17 patients withdrew consent after randomisation. Even though these patients were equally distributed among the three randomisation groups, the acceptability rate of peg-IFN treatment was low, and similar to previous studies.\textsuperscript{28} Furthermore, judging by the high rate of adverse events and treatment discontinuation, peg-IFN was not well tolerated.

In this study, we have included two groups of combination therapy. In addition to its antiviral effect, adefovir has been shown to enhance innate immune functions, suggesting a possible synergistic effect when combined with peg-IFN.\textsuperscript{34,35} In our previous study, peg-IFN plus adefovir combination therapy resulted in high rates of HBsAg loss. Because adefovir has largely been replaced by tenofovir, which has a similar mechanism of action but is more potent in suppressing hepatitis B virus DNA, we also included a group with peg-IFN plus tenofovir combination.\textsuperscript{31} Because there are no previous data for treatment of patients with a low viral load, comparison of our results with peg-IFN or nucleos(t)ide analogue monotherapy data is not possible. However, we aimed to investigate the difference between combination therapy and no treatment. Based on natural history reports, HBsAg loss in untreated patients with chronic hepatitis B is 0.8–1.0% per year, which makes a control group indispensable.\textsuperscript{36,37}

In conclusion, to our knowledge, this is the first treatment intervention study done in patients with chronic hepatitis B with a low viral load. Although HBsAg clearance rates did not differ significantly between the treatment and control groups, HBsAg decline was significantly greater in patients treated with peg-IFN and nucleos(t)ide analogue combination therapy at week 72 than in untreated patients. Although follow-up
data will show whether a strong HBsAg decline could lead to higher rates of HBsAg loss in the treated patients in the long term, our findings do not support the use of combination treatment with peg-IFN and nucleos(t)ide analogue in patients with chronic hepatitis B with a low viral load. Pursuing functional cure for patients with chronic hepatitis B with a low viral load is supported by various benefits for the patient, including the discontinuation of close monitoring, a decreased risks of hepatitis B virus reactivation, and overcoming a stigma. The development of new antiviral hepatitis B virus compounds gives hope for future treatment for this large group of patients with chronic hepatitis B.

ACKNOWLEDGEMENTS

We would like to express great appreciation to Martine W Peters, Jeltje Helder, and Hadassa Heidsieck for patient follow-up and sampling. We thank Marjan J Sinnige for handling patient material and doing additional immunological analyses, as well as Meike H van der Ree for critical revision of the manuscript.

SUPPLEMENTARY DATA

Supplementary data associated with this article available can be found in the online version of this article.
REFERENCES


CHAPTER 4

IP-10 in Chronic Hepatitis C Patients Treated with High-Dose Interferon


The Netherlands Journal of Medicine 2014; 72(8): 407–415
ABSTRACT

Introduction
Interferon-γ-Inducible-Protein-10 (IP-10) serum levels are associated with IL28B genotype and may predict response to interferon/ribavirin-based therapy in chronic hepatitis C patients. Our aim was to relate IP-10 levels before and during treatment to treatment outcome, viral HCV-RNA kinetics and IL28B genotype.

Patients and methods
A cohort of chronic hepatitis C patients was treated for 6 weeks with high-dose interferon, followed by standard peginterferon/ribavirin for 24 or 48 weeks. IP-10 and HCV-RNA levels were frequently determined before, during and after treatment.

Results
IP-10 levels increased from log2.56 at baseline to log3.48 pg/mL at Day1 and diminished thereafter gradually. IP-10 levels at any time point were not statistically different between patients with or without SVR. Patients with IL28B CC genotype had significantly lower baseline IP-10 levels (p = 0.019) and a higher increase of IP-10 levels from baseline to Day1 than patients with IL28B non-CC genotypes (p = 0.015). Patients with HCV-RNA decline ≥2.28log10 at Day1 had significantly lower baseline IP-10 levels (p=0.016) and a higher increase of IP-10 levels from baseline to Day1 (p = 0.047) than patients with HCV-RNA decline of < 2.28log10 at Day 1.

Conclusions
In patients treated with high induction-dose interferon, IP-10 levels at any time point were not predictive for SVR. Low baseline IP-10 levels and a higher increase of IP-10 levels from baseline to Day1 were associated with IL28B CC genotype and HCV-RNA decline ≥2.28log10 at Day 1. This suggests that for prediction of SVR in our cohort the added value of IP-10 to IL28B genotype and early viral kinetics is limited.
INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis affecting over 170 million people worldwide.\(^1\) After being exposed to HCV, a chronic infection develops in approximately 80% of cases.\(^2\) Chronic hepatitis C (CHC) is characterized by liver inflammation due to pro-inflammatory cytokines and infiltration of specific and non-specific T-lymphocytes.\(^3\) The damage inflicted leads to liver fibrosis and may ultimately cause liver cirrhosis, hepatocellular carcinoma and death.\(^3\)

After an infection with HCV the innate immune system initiates a nonspecific immune response through type I interferon, leading to the activation of the intracellular pathway resulting in the induction of multiple interferon-stimulated genes (ISG's). Type I interferon has also immunomodulatory effects by activating and modulating the function of different kinds of leukocytes, including natural killer (NK) cells, macrophages, dendritic cells (DC) and T-lymphocytes. This results in a strong specific CD4+/CD8+ T-cell response leading ideally to the clearance of HCV.\(^4\) In most cases however, a chronic HCV infection is established, in which the HCV specific immune responses are weaker and less specific than in acute resolving HCV infection.\(^5\)

The gene encoding the non-ELR CXC chemokine interferon-\(\gamma\)-inducible protein-10 (IP-10 or CXCL10) is an ISG that is induced by interferon-\(\gamma\) and tumor necrosis factor (TNF) alpha. It is produced by different kinds of cells such as endothelial cells, fibroblasts, mesangial cells, monocytes, neutrophils and hepatocytes. After binding to its receptor CXCR3, IP-10 functions as a chemotactic cytokine for T-lymphocytes, monocytes and NK-cells and induces adhesion of activated memory/effecter T-cells.\(^6\) Levels of IP-10 are higher in patients with chronic HCV infection than in healthy controls.\(^7\)

Multiple inflammatory chemokines and cytokines have been suggested as markers for treatment outcome because of their regulatory function in the HCV-specific immune response. Most of these cytokines are modulated by exogenous interferon and play a critical role in viral clearance.\(^8\) In patients who develop a sustained viral response (SVR) after interferon-based therapy, the baseline activation of the immune system tends to be lower prior to treatment than in patients who do not achieve SVR.\(^8\)\(^–\)\(^10\) This difference of baseline activation of the immune system might be influenced by single nucleotide polymorphisms (SNP's) on chromosome 19 near the interleukin-28B gene (IL28B), encoding interferon-\(\lambda\). IL28B gene polymorphisms are highly associated with treatment outcome in CHC patients treated with interferon-based therapy.\(^11\) Most data have been published on two of these gene polymorphisms, SNP's rs12979860 and rs8099917, associated with SVR after peginterferon and ribavirin therapy.\(^12,13\)

Baseline IP-10 levels may be a prognostic marker for the outcome of interferon-based therapy in HCV infection.\(^14,23\) There are several studies that describe a relation between low baseline IP-10 levels and higher rates of rapid viral response (RVR, HCV-RNA undetectable after 4 Weeks of treatment)\(^14,15,20,23\) and SVR after treatment with peginterferon and ribavirin.\(^14\)\(^–\)\(^22\) However, whether the IP-10 level really is a predictor for SVR and/or RVR remains a subject of discussion.

From 2002 to 2005 a cohort of CHC patients (treatment naïve patients with HCV genotype 1 or 4 and patients of all genotypes with failure to interferon-based therapy) was treated with a high induction dose of interferon combined with ribavirin,
followed by peginterferon and ribavirin. Our aim was to investigate in this cohort of patients whether IP-10 levels before and during treatment with this high-dose of interferon were related to treatment outcome, IL28B genotype and HCV-RNA kinetics.

**PATIENTS AND METHODS**

**Patients and treatment regimen**
From 2002 to 2005, a cohort-study was performed in which 100 CHC patients were included (treatment naïve patients with HCV genotype 1 or 4 and patients of all genotypes who failed previous therapy with either classical interferon alone, or a combination of (peg)interferon and ribavirin). Results of this study have been reported in 2008. All patients were treated for 6 weeks with high-dose interferon-alpha 2b (Merck Pharmaceuticals, USA), combined with ribavirin (weight-based: 1000 mg/day in patients weighing <75 kg, and 1200 mg/day in patients weighing >75 kg), followed by 24 or 48 weeks of peginterferon alpha 2b (1.5 ug/kg once a week) and ribavirin (weight-based 1000–1200 mg/day). All patients were also treated with amantadine hydrochloride 200 mg/day (Symmetrel®, Novartis, Basel, Switzerland). Figure 1. describes the study design.

During the first six weeks of treatment the following interferon-induction scheme was used: Weeks 1 and 2: 18 MU/ day in three divided doses; Weeks 3 and 4: 9 MU/d in 3 divided doses; Weeks 5 and 6: 6 MU/d in 2 divided doses. Patients with a decline in HCV-RNA ≥3log10 at Week 4 (and TMA-undetectable at week 24) were randomized to stop treatment at 24 Weeks or to continue to 48 weeks. Patients with a decline in HCV-RNA <3log10 at Week 4 were treated for 48 Weeks. Treatment was stopped in all patients with detectable HCV-RNA at Week 24. All patients were followed for 24 Weeks after completion of therapy.

Plasma samples were stored at -80°C at baseline, Days 1 and 3, Weeks 1, 2, 3, 4, 6, 8, every 4 weeks until the end of treatment, and after cessation at Weeks 4, 12 and 24. The study was approved by the institutional review board. Written informed consent was obtained from each patient.

**Patient and sample selection for measurements**
All patients who completed the whole treatment course or who had to stop treatment before Week 24 or 48 because of stopping criteria, were included in our study to determine IL28B genotype and to measure IP-10 and HCV-RNA levels at baseline, Day 1, Week 1, 2, 4 and 6, at end of treatment (EOT) and at end of follow-up (EFU). Patients who stopped treatment prematurely (dropouts) between Day 0 and Week 24 (for other reasons than the above mentioned stopping criteria), and patients of whom baseline plasma samples were not available were excluded. Of the 100 included patients in the original study, 85 patients were included in this study. Reasons for exclusion of the remaining 15 patients were drop-out due to side effects of the treatment (n = 12), dropout because of non-medical reasons (n = 1), and lack of available plasma samples (n = 2). From six of the included 85 patients Day 1 plasma samples were missing. For that reason, change in IP-10 levels from baseline to Day 1 could not be calculated and therefore these patients were excluded.
HCV-RNA measurement
HCV-RNA was quantitatively measured using a bDNA assay (VERSANT® HCV 3.0 assay; Siemens, Germany); linear dynamic range $6.15 \times 10^2$ to $7.7 \times 10^6$ IU/mL. A qualitative HCV-RNA measurement was performed when the quantitative test was negative, using transcription-mediated amplification (TMA) (VERSANT® HCV qualitative assay, Siemens, Germany; lower limit of detection (LLD) 5 IU/mL). HCV genotypes were determined using the TruGene® HCV genotyping assay and the Open-Gene® automated DNA sequencing system (Bayer Diagnostics, Berkeley, California, USA).

IP-10 measurement
IP-10 levels were measured using a solid base sandwich ELISA (lower limit of detection 4.46 pg/ml, dynamic quantitative assay range 7.8 – 500 pg/mL; Quantikine human CXCL10/IP-10 immunoassay, R&D Systems). Plasma samples were tested in duplicate in a dilution of 1:5 (according to manufacturer’s description). A first evaluation of the test results showed that in many cases IP-10 levels, especially at Day 1, were above the upper limit of the assay range of 500 pg/mL. By using Bland-Altman plots comparing duplicate measurements, we retested all plasma samples with an initial test value >730 pg/mL (with 1:5 dilution) after a second dilution step of 1:5, resulting in a dilution of 1:25 for calculation of IP-10 levels.

IL28B genotyping
IL28B single nucleotide polymorphism (SNP) genotyping (rs12979860) was performed by High Resolution Melting Curve Analysis (HRMCA) on a LightCycler480 (Roche Applied Science) using custom-designed primers and LC480 High Resolution Melting Master (Roche Applied Science). Results were analyzed with the LC480 HRMCA module implemented in the LC480 Software.
Assessment of treatment outcome

The following definitions were used to categorise treatment outcomes:

SVR: Undetectable HCV-RNA at end of follow-up (24 Weeks after end of treatment);
RVR: Undetectable HCV-RNA at Week 4 during treatment; Non-response: Detectable HCV-RNA (TMA positive) at all time points during treatment and at end of follow-up; Relapse: Undetectable HCV-RNA (TMA negative) at end of treatment but detectable HCV-RNA at end of follow-up; Non-SVR: All patients who did not achieve SVR. Dropout: Any patient who stopped treatment prematurely between Day 0 and Week 24/48 or who was lost to follow up during 24 Weeks thereafter.

Statistical analysis

IP-10 values were logarithmically transformed to achieve a normal distribution. Graphic representation was performed using Graphpad Prism version 5 for Windows (GraphPad Software, San Diego, California, USA) and SPSS version 19.2 for Windows (SPSS Inc., Chicago, Illinois, USA). Data were analyzed on per protocol basis. We used the Bland-Altman plots, Student’s t-test, the Mann-Whitney U-test, chi-square and Fisher’s Exact test where appropriate. Differences were considered statistically significant when p was < 0.05. A receiver operating characteristic (ROC)-analysis was performed to determine at Day 1 which level of HCV-RNA decline gave the best prediction for SVR.

RESULTS

Baseline characteristics and treatment outcome

Baseline characteristics of the 85 patients included in the study are shown in Table 1. Thirty-six of the 85 patients (42%) achieved SVR, whereas 49 (58%) did not. Treatment naïve patients, patients with RVR, IL28B CC genotype or a low METAVIR fibrosis stage (F0-F1-F2) were significantly more likely to achieve SVR. The group of patients with genotype 2, 3 or 5 had a higher SVR rate than patients with genotype 1 or 4. Statistically this was not significant, but there was a trend (p = 0.09). IP-10 levels at baseline were lower in patients with SVR compared to patients without SVR, but this difference was not statistically significant (Table 1.). There was no statistically significant difference in baseline IP-10 levels between patients with partial response or patients with null response (data not shown). There were 26 patients with IL28B genotype CC of which 17 (65%) had SVR and 9 (35%) did not. Of the 59 IL28B non-CC genotype patients 19 (32%) had SVR (p = 0.008) (Table 1.).

A cut-off of < / ≥ 600 pg/mL was used (chosen based on earlier literature16) to define high and low IP-10 levels at baseline. In the group of patients with baseline IP-10 levels ≥ 600 pg/mL. Treatment-experienced patients had lower SVR-rates that treatment-naïve patients. However, these differences were not statistically significant (Table 2.).

Baseline IP-10 levels and response parameters

Mean log IP-10 levels at baseline were significantly lower in patients achieving RVR than in patients without RVR (2.43 pg/mL / 2.62 pg/mL, p = 0.016) (Table 3.). This was also the case in patients with IL28B CC genotype versus patients with IL28B non-CC
Table 1. Baseline characteristics of patients treated with high-dose induction interferon followed by peginterferon and ribavirin for 24 or 48 weeks according to SVR

<table>
<thead>
<tr>
<th></th>
<th>SVR</th>
<th>Non-SVR</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>36 (42)</td>
<td>49 (58)</td>
<td></td>
</tr>
<tr>
<td>Male (%)/ female (%)</td>
<td>28 (33)/ 8 (9)</td>
<td>38 (46)/ 11 (13)</td>
<td>0.98</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>44 (25 – 63)</td>
<td>46 (19 – 67)</td>
<td>0.37</td>
</tr>
<tr>
<td>Baseline HCV-RNA (log)</td>
<td>5.97</td>
<td>5.77</td>
<td>0.28</td>
</tr>
<tr>
<td>Naïve / non-naïve (%) *</td>
<td>24 (28)/ 12 (14)</td>
<td>22 (26)/ 27 (32)</td>
<td>0.046</td>
</tr>
<tr>
<td>Genotype (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>23 (27)</td>
<td>34 (40)</td>
<td>0.65</td>
</tr>
<tr>
<td>4</td>
<td>6 (7)</td>
<td>12 (14)</td>
<td>0.43</td>
</tr>
<tr>
<td>2/3/5</td>
<td>7 (8)</td>
<td>3 (4)</td>
<td>0.09</td>
</tr>
<tr>
<td>RVR / non-RVR (%)</td>
<td>19 (22)/ 17 (20)</td>
<td>5 (6) / 44 (52)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL28B genotype CC / non-CC (%)</td>
<td>17 (20) / 19 (23)</td>
<td>9 (11) / 40 (47)</td>
<td>0.008</td>
</tr>
<tr>
<td>Baseline IP-10 (log pg/mL) (+/- SEM)</td>
<td>2.53 (0.04)</td>
<td>2.59 (0.05)</td>
<td>0.34</td>
</tr>
<tr>
<td>Liver biopsy (%)</td>
<td>32 (41)</td>
<td>46 (59)</td>
<td></td>
</tr>
<tr>
<td>Fibrosis stage Metavir F3/F4 (%)</td>
<td>12 (15)</td>
<td>31 (40)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Non-naïve: earlier treatment with either classical interferon alone, or combination therapy with (peg)interferon and ribavirin

Table 2. SVR versus non-SVR in naïve and treatment experienced patients with baseline IP-10 levels of < or ≥ 600 pg/mL

<table>
<thead>
<tr>
<th>IP-10 baseline (pg/mL)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 600 pg/mL</td>
</tr>
<tr>
<td>Naïve</td>
<td>19/38 (50)</td>
</tr>
<tr>
<td>Non-naïve</td>
<td>10/32 (31)</td>
</tr>
<tr>
<td>SVR</td>
<td>39/70 (54)</td>
</tr>
<tr>
<td>Total</td>
<td>39/70 (54)</td>
</tr>
</tbody>
</table>

genotypes (2.45 pg/mL / 2.62 pg/mL, p = 0.019) (Table 3). Statistically there was a trend towards lower baseline mean log IP-10 levels in HCV genotype non-1 patients (compared to HCV genotype 1 patients, p = 0.098) (Table 3). For all other parameters shown in Table 3. there was no statistically significant difference in baseline IP-10 levels. Because it is well-known that IP-10 levels and IL28B are related, we performed a multivariate analysis showing that IL28B CC genotype was an independent predictor of RVR (Table 4.). This multivariate analysis showed a trend towards lower baseline IP-10 levels in patients achieving RVR (p = 0.079).
### Table 3. Baseline IP-10 levels and various response parameters

<table>
<thead>
<tr>
<th></th>
<th>Baseline IP-10 levels (mean log +/- SEM, pg/mL)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve / non-Naïve</td>
<td>2.54 (0.05) / 2.59 (0.04)</td>
<td>0.51</td>
</tr>
<tr>
<td>Genotype 1 / Genotype non-1</td>
<td>2.60 (0.04) / 2.50 (0.04)</td>
<td>0.098</td>
</tr>
<tr>
<td>Baseline HCV-RNA &lt; 600.000 / ≥ 600.000 IU/mL</td>
<td>2.57 (0.05) / 2.56 (0.04)</td>
<td>0.81</td>
</tr>
<tr>
<td>Fibrosis score Metavir F3-F4 / F0-F2</td>
<td>2.56 (0.04) / 2.58 (0.05)</td>
<td>0.81</td>
</tr>
<tr>
<td>IL28B genotype CC / non-CC</td>
<td>2.45 (0.05) / 2.62 (0.04)</td>
<td>0.019</td>
</tr>
<tr>
<td>RVR / non-RVR</td>
<td>2.44 (0.05) / 2.61 (0.04)</td>
<td>0.016</td>
</tr>
</tbody>
</table>

### Table 4. Predictors of RVR: multivariate analysis of baseline IP-10 levels and IL28B genotype

<table>
<thead>
<tr>
<th></th>
<th>RVR</th>
<th>Non-RVR</th>
<th>Confidence Interval (95%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL28B genotype CC, N (%)</td>
<td>14</td>
<td>12</td>
<td>0.78 – 0.65</td>
<td>0.006</td>
</tr>
<tr>
<td>non-CC, N (%)</td>
<td>10</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log IP-10 baseline (mean, pg/mL)</td>
<td>2.44</td>
<td>2.61</td>
<td>0.013 – 1.267</td>
<td>0.079</td>
</tr>
<tr>
<td>Total, N (%)</td>
<td>24</td>
<td>61</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 5. Factor of increase in IP-10 levels from baseline to Day 1, different baseline IP-10 levels (dependent on the baseline IP-10 level: the lower the baseline IP-10 level, the higher the factor of increase).

<table>
<thead>
<tr>
<th>IP-10 baseline</th>
<th>N</th>
<th>Factor of increase D1 (mean)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 150</td>
<td>8</td>
<td>27</td>
<td>0.005</td>
</tr>
<tr>
<td>≥ 150</td>
<td>71</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>&lt; 300</td>
<td>31</td>
<td>16</td>
<td>0.001</td>
</tr>
<tr>
<td>≥ 300</td>
<td>48</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>&lt; 375</td>
<td>41</td>
<td>15</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≥ 375</td>
<td>38</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>&lt; 600</td>
<td>68</td>
<td>13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≥ 600</td>
<td>11</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

### Table 6. Patients with a decline of HCV-RNA at Day 1 of ≥ or < 2.28log10 and SVR or non-SVR status.

<table>
<thead>
<tr>
<th>Decline HCV-RNA Day 1</th>
<th>SVR (N)</th>
<th>Non-SVR (N)</th>
<th>Total</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 2.28 log₁₀</td>
<td>21</td>
<td>7</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2.28 log₁₀</td>
<td>15</td>
<td>42</td>
<td>57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>49</td>
<td>85</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PPV = positive predictive value; NPV = negative predictive value; Sens = sensitivity; Spec = specificity
Figure 2. IP-10 levels before and during treatment. An 1 log10 rise at Day 1 was observed, and thereafter IP-10 levels gradually declined and were significantly lower than baseline levels at end of treatment (EOT) and end of follow-up (EFU). * p = 0.01 ** p = 0.01

Figure 3. IP-10 levels before and during treatment: SVR versus non-SVR EOT at 24 Weeks (n=55) or 48 Weeks (n=30); EFU at 24 Weeks after EOT (n=85). * p < 0.001
From baseline to Day 1 an almost 10-fold increase of mean log IP-10 levels was observed (from log 2.56 pg/mL to log 3.48 pg/mL) (Figure 2.). The range of the fold increase in IP-10 levels was 2 to 40. The increase was related to baseline IP-10 levels: the lower the baseline IP-10 levels, the greater the increase at Day 1 (Table 5.). Thereafter, mean log IP-10 levels diminished gradually, returning to baseline levels between Week 4 and 6 of treatment, and diminishing further to a level significantly lower than the baseline level at EOT (2.41 pg/mL, p = 0.01) and EFU (2.35 pg/mL, p = 0.01) (Figure 2.).

**IP-10 levels during therapy**
From baseline to Day 1 an almost 10-fold increase of mean log IP-10 levels was observed (from log 2.56 pg/mL to log 3.48 pg/mL) (Figure 2.). The range of the fold increase in IP-10 levels was 2 to 40. The increase was related to baseline IP-10 levels: the lower the baseline IP-10 levels, the greater the increase at Day 1 (Table 5.). Thereafter, mean log IP-10 levels diminished gradually, returning to baseline levels between Week 4 and 6 of treatment, and diminishing further to a level significantly lower than the baseline level at EOT (2.41 pg/mL, p = 0.01) and EFU (2.35 pg/mL, p = 0.01) (Figure 2.).

**IP-10 levels during therapy and treatment outcome**
Before and during treatment mean log IP-10 levels were in general lower in SVR patients than in non-SVR patients, but this difference was not statistically significant at any time point (Figure 3.). At EFU, mean log IP-10 levels were significantly lower in patients with SVR than in non-SVR patients (2.40 pg/mL versus 2.43 pg/mL, p<0.0001) (Figure 3.).
IP-10 levels during therapy and IL28B genotype
The increase of IP-10 levels from baseline to Day 1 was significantly greater in patients with IL28B CC genotype than in patients with IL28B non-CC genotypes (log1.07 pg/mL versus log0.89 pg/mL, p=0.015) (Table 7).

DISCUSSION
In contrast to what has been described earlier, we did not find a clear association between IP-10 levels before or during treatment and SVR or non-SVR. There are also other studies that, like ours, did not confirm the association between a low baseline IP-10 level and SVR. Nevertheless, in our study baseline IP-10 levels were significantly lower in patients with RVR than in those without RVR. The association of RVR and low baseline IP-10 levels without a significant difference in baseline IP-10 between SVR and non-SVR patients was previously described in HCV genotype 1 and 4 patients and in patients with acute HCV infection. However, there are reports contradicting these findings, in which no difference was seen in baseline IP-10 levels between CHC patients with or without RVR, or with or without SVR. We also found a clear relation between IL28B genotype and SVR, in line with previous data.

A possible explanation for the relationship we observed between baseline IP-10 levels and RVR and the absence of a relationship between baseline IP-10 levels and SVR may be that the high induction dose of interferon resulted in a higher rate of RVR than would have occurred with standard dose of IFN. Consequently, this higher rate of RVR with high induction IFN may not have the same predictive value for SVR as with standard (peg)IFN. It may also be that our cohort of patients was too small to show a statistical difference in baseline IP-10 levels and change in IP-10 levels during treatment between patients achieving SVR or not. In multivariate analysis the association we found between low baseline IP-10 levels and RVR seemed to be dependent on IL28B CC genotype, where IL28B CC genotype was an independent predictor of RVR. This suggests that IL28B genotype is a more important factor for prediction of RVR (and SVR) than baseline IP-10 levels.

Our findings, demonstrating a relation between IL28B genotype and IP-10 levels, confirm the results of earlier studies, showing that patients with favourable IL28B polymorphisms (CC) had lower pre-treatment IP-10 levels than patients with unfavourable IL28B genotypes (CT or TT). These studies also showed that when pre-treatment IP-10 levels are low (<600 pg/mL), the predictive value for RVR or SVR of IL28B genotype is increased (especially in patients with CT and TT genotypes). These findings, together with ours, implicate the utility of combining these two markers in predicting treatment outcome. Also in patients with acute HCV infection low serum IP-10 levels increased the predictive value of IL28B polymorphisms (SNPs rs12979860 and rs8099917) with regards to the spontaneous clearance of HCV.

Treatment experienced patients had a lower SVR-rate than treatment naïve patients. In patients with a baseline IP-10 level of ≥600 pg/mL, SVR rate was lower than in patients with a baseline IP-10 level of < 600 pg/mL, especially in treatment experienced patients. These differences were not statistically significant, but numbers were very small (n=13). These findings confirm, what was already known, that treatment experienced patients...
were less interferon-responsive than naïve patients. The higher dose of interferon did not overcome this irresponsiveness. The fact that we did not find a relation between baseline IP-10 levels and SVR, and the fact that in this cohort of patients SVR-rates were not higher than SVR-rates of comparable cohorts of patients treated with standard peginterferon and ribavirin therapy, as described in literature\textsuperscript{36–40}, supports this.

Our study is the first to describe IP-10 kinetics in CHC patients treated with high-dose interferon and amantadine. We found an almost 10-fold increase of IP-10 levels at Day 1 after the start of treatment, which was dependent of baseline IP-10 levels (4-fold when baseline IP-10 level was \( \geq 600 \) pg/mL to 27-fold when baseline IP-10 level < 150 pg/mL). A rise in IP-10 levels dependent of baseline IP-10 levels shortly (24 hours) after the start of treatment with peginterferon and ribavirin was also described in HCV/HIV co-infected patients.\textsuperscript{32} In this study a 3-fold rise was seen in patients with a baseline IP-10 level of > 600 pg/mL versus a 9-fold rise in patients with a baseline IP-10 level of < 150 pg/mL. Another study showed a dose-dependent 2- to 5-fold rise in IP-10 level, 2 days after the start of a low dose versus a normal dose of peginterferon in CHC patients.\textsuperscript{30} As interferon up-regulates ISG’s, including IP-10, one may expect that the IP-10 expression induced after a high dose of interferon is greater than after a lower dose. Our data support this suggestion, and it may be that high-dose interferon induces such a high level of IP-10 expression that other factors such as the baseline IP-10 level are less important as a predictor for RVR and SVR.

We also found that, after the initial rise, of IP-10 levels, the levels gradually declined to below the baseline value at end of treatment and at end of follow-up, and was significantly lower in patients achieving SVR. This was previously described,\textsuperscript{20,22,28} and may indicate that when HCV-RNA levels are declining, IP-10 is down-regulated.

It is unlikely that the addition of amantadine to the treatment regimen of our cohort of patients did influence SVR and IP-10 levels, since SVR rates were not different in patients with or without addition of amantadine, as was shown in several studies.\textsuperscript{41,42}

In our study, a first phase viral decline (HCV-RNA decline of \( \geq 2.28 \log_{10} \) at Day 1) was associated with lower baseline IP-10 levels, which is supported by earlier studies.\textsuperscript{18,32} One of these studies showed that a first phase decline of HCV-RNA of \( > 1 \log_{10} \) at Day 1 of treatment with peginterferon/ribavirin was associated with lower IP-10 levels at baseline.\textsuperscript{18} In HIV/HCV co-infected patients a similar pattern has been described, with a negative correlation between baseline IP-10 levels and the degree of HCV-RNA decline at Day 2 of treatment with peginterferon/ribavirin.\textsuperscript{32} In contrast to earlier experience with interferon-based therapy, one study with peginterferon monotherapy combined with danoprevir showed that baseline IP-10 levels were positively correlated with a decline of HCV-RNA at Day 1 of treatment and that IP-10 levels at Day 7 and Day 14 were significantly lower than at baseline.\textsuperscript{35} The association we found between this large first phase decline of HCV-RNA \( \geq 2.28 \log_{10} \) and a significantly higher increase of IP-10 levels from baseline to Day 1 of treatment has not been described before. This may be due to the high induction dose of interferon applied in our study, inducing strong up-regulation of ISG’s responsible for a rapid decline of HCV-RNA. Our finding that the increase of IP-10 levels from baseline to Day 1 was larger in patients with IL28B CC genotype than in IL28B non-CC patients, suggests that induction of IP-10 is dependent of the IL28B genotype. This is also supported by our findings in multivariate analysis, where IL28B CC genotype was an independent predictor of RVR, but baseline IP-10 level was not.
A limitation to our study is the fact that our data were valid for patients with HCV genotype 1 and 4 because only limited numbers of patients with genotype 2, 3 and 5 were included in our study.

In conclusion, there was no significant difference in IP-10 levels between patients with or without SVR, but baseline IP-10 level was significantly lower in patients with RVR versus non-RVR. IP-10 levels changed markedly after one day of treatment with high induction-dose interferon. The factor of increase of IP-10 levels from baseline to Day 1 was higher, when the baseline IP-10 level was lower. There was a clear relation between IP-10 levels at baseline and Day 1 of treatment and a decline of HCV-RNA of $\geq 2.28\log_{10}$ at Day 1. Baseline and dynamic IP-10 levels early during treatment seem to be closely related to early viral kinetics and IL28B genotype. At present all-oral DAA combination treatment will result in eradication of HCV in most patients, and predictive markers for response become of less importance. However, in the future some patients like HCV genotype 3 and some difficult-to-treat patients such as end-stage liver cirrhotics will fail to achieve SVR. Immunological markers may help to understand why some patients fail also with DAA therapy.

ACKNOWLEDGEMENTS

We acknowledge professor C.P. Engelfriet for critical review of our manuscript.
REFERENCES


CHAPTER 5

Sofosbuvir plus Simeprevir for the Treatment of HCV Genotype 4 Patients with Advanced Fibrosis or Compensated Cirrhosis is Highly Efficacious in Real-Life


Journal of Viral Hepatitis 2016; 23(12): 950–954
ABSTRACT

Introduction
Chronic hepatitis C virus (HCV) infection is a major cause of chronic liver disease and liver-related death. Recently, multiple regimens of different direct-acting antiviral agents (DAA’s) have been registered. Although treatment with sofosbuvir (SOF) and simeprevir (SMV) is registered for the treatment of genotype 4 patients in some countries, data on efficacy of this combination is lacking. We aimed to assess the efficacy of SOF and SMV with or without RBV during 12 weeks in a real-life cohort of genotype 4 HCV patients.

Patients and Methods
A retrospective multi-center observational study was conducted in 4 hospitals in Amsterdam, the Netherlands, including patients with advanced liver fibrosis or liver cirrhosis treated with SOF plus SMV with or without RBV during 12 weeks for a genotype 4 chronic HCV infection from 1/1/2015 to 1/8/2015. Sustained Viral Response (SVR) was established at week 12 after end of treatment.

Results
A total of 53 patients with genotype 4 HCV infection, treatment naïve and experienced, were included. SVR was achieved in 49/53 patients (92%). The four failures all had a virological relapse and did not receive ribavirin. Three were non-responder to earlier interferon-based treatment and one was treatment-naive.

Conclusions
In this real-life cohort of patients with HCV genotype 4 infection and advanced liver fibrosis/cirrhosis we show that treatment with SOF and SMV is effective. The addition of RBV could be considered in treatment-experienced patients as recommended in guidelines.
INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major cause of chronic liver disease. It causes liver fibrosis and may ultimately lead to liver cirrhosis, hepatocellular carcinoma and death.\(^1\) It is estimated that there are around 80 million people worldwide with chronic HCV infection.\(^2\)

Recently, new potent all-oral antiviral treatment regimens resulting in very high cure rates in all HCV genotypes were registered. For patients with hepatitis C genotype 4 the combination of sofosbuvir (SOF) and simeprevir (SMV) is recommended in European guidelines as one of the treatment indications.\(^3\) Although various studies in genotype 1 chronic hepatitis C (CHC) patients reported sustained viral response (SVR) rates of > 90% after treatment with SOF and SMV for 12 weeks with or without ribavirin (RBV), data in genotype 4 CHC are lacking.\(^4,5\) This recommendation in the guidelines is based on study results from SOF or SMV combined with both peginterferon (PegIFN) and RBV and SOF/RBV showing similar results in HCV genotype 1 and 4 patients.\(^6-12\) EASL recommendations state that given the effectiveness of both SOF and SMV against HCV genotype 4, it is likely that the results of combination therapy with SOF and SMV in HCV genotype 1 patients can be extrapolated.\(^3\) This statement has also been followed in the Dutch guidelines.\(^13\) Whether RBV should be added to this treatment regimen is unknown. Recent studies in HCV genotype 1 cirrhotic patients treated with SOF/SMV with or without ribavirin, contradicted each other in the value of the addition of RBV.\(^14,15\)

Our aim was to assess in real-life the efficacy of combination treatment of SOF and SMV with or without RBV during 12 weeks for HCV genotype 4 patients.

PATIENTS AND METHODS

We conducted a retrospective multi-center observational study in two large University Hospitals and two large general hospitals in Amsterdam, the Netherlands. We included all HCV genotype 4 patients treated with SOF plus SMV with or without RBV initiating treatment from January until August 2015 (treatment-naïve and interferon treatment-experienced patients with advanced fibrosis or compensated liver cirrhosis (FibroScan ≥ 9.5 kPa or liver biopsy fitting with fibrosis score). All patients were treated with a combination of SOF (400 mg orally once daily) and SMV (150 mg orally once daily) with or without RBV at the physician’s discretion (weight-based upon body weight: < 80 kg 1000 mg per day; ≥ 80 kg 1200 mg per day, split in two doses daily) for 12 weeks.

Sustained viral response was defined as undetectable HCV-RNA 12 weeks after end of treatment. Rapid virological response (RVR) at Week 4 after start of treatment was documented. Different clinical (sex, age, treatment experience, medical history, co-medications, fibroscan results) and laboratory parameters (haematology, chemistry, haemostasis, virology) were documented during and after treatment: at Baseline (BL), Week 4 (W4), Week 12 (end of treatment, EOT) and 12 Weeks after EOT (end of follow-up, EFU). Liver cirrhosis was defined as FibroScan ≥ 14.0 kPa or liver biopsy fitting with liver cirrhosis (Ishak score ≥ 5 \(^16\) or METAVIR score F4 \(^17,18\)).
HCV-RNA measurement
HCV-RNA was quantitatively measured using the Roche Cobas Ampliprep-Cobas Taqman (lower limit of detection 5 IU/mL; Hoffman-LaRoche, Basel, Switzerland)\(^9\) or the Abott RealTime HCV assay (lower limit of detection 12 IU/mL; Abbott Diagnostics, Lake Forest, IL, USA).\(^{20}\)

Assessment of treatment outcome
The following definitions were used to categorise treatment outcomes:
- SVR: Undetectable HCV-RNA 12 weeks after EOT;
- RVR: Undetectable HCV-RNA at Week 4 during treatment;
- Virological Relapse: Undetectable HCV-RNA at end of treatment but detectable HCV-RNA at Week 12 after EOT;
- Non-SVR: All patients who did not achieve SVR.

Statistical analysis
Graphic representation was performed using Graphpad Prism\textsuperscript{®} version 6 for Windows (GraphPad Software, San Diego, California, USA) and SPSS version 22 for Windows (SPSS Inc., Chicago, Illinois, USA). We used the Student’s \(t\)-test, the Mann-Whitney U-test, chi-square and Fisher’s Exact test where appropriate. Differences were considered statistically significant when \(p < 0.05\).

RESULTS

A total of 53 HCV genotype 4 patients initiated treatment with SOF/SMV. All patients were treated for 12 weeks. Table 1. shows the baseline characteristics of all treated patients.

Fourty-one (77\%) were treated without RBV and 12 (23\%) with RBV. Of the 53 included patients, 49 achieved SVR (92\%). The four failures all had a virological relapse and did not receive ribavirin. Three out of the four failures were non-responder to previous interferon-based therapy, and one was treatment-naive. Three out of 4 failures were cirrhotic patients, of which one had severe thrombocytopenia (< 90 × 10\(^E9\)/L) as a sign of portal hypertension. Figure 1. shows SVR according to cirrhosis, RBV-use, prior treatment experience and thrombocytopenia.

All patients with cirrhosis had Child-Pugh score A. There was no relationship between HCV-RNA at week 4 and non-SVR. Diabetes Mellitus Type 2, hypertension and dyslipidemia were frequent comorbidities. The use of co-medication was frequent (mainly consisted of statins, antidiabetics, and antihypertensive medication), but none of the patients used co-medication known to interact with SOF or SMV. Of the four patients with virological relapse, one patient used citalopram for depression, two patients used an ACE inhibitor for hypertension, one patient used pantoprazole and one patient used no co-medication. In the group treated with RBV, relatively more patients had thrombocytopenia (< 90 × 10\(^E9\)/L) and were treatment experienced compared to the group treated without RBV prior to the initiation of treatment. Table 2. shows characteristics of the patients according to the addition of RBV to the treatment regimen.
Table 1. Baseline characteristics of patients treated with SOF and SMV +/- RBV according to SVR.

<table>
<thead>
<tr>
<th></th>
<th>SVR</th>
<th>Non-SVR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>49 (92%)</td>
<td>4 (8%)</td>
<td></td>
</tr>
<tr>
<td>Male/female (N)</td>
<td>46/3</td>
<td>4/0</td>
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</tr>
<tr>
<td>Age at start (years, median) (range)</td>
<td>52 (37-68)</td>
<td>50 (38–54)</td>
<td>NS</td>
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<tr>
<td>RBV +/- (N)</td>
<td>12/37</td>
<td>0/4</td>
<td>NS</td>
</tr>
<tr>
<td>Baseline HCV-RNA (IU/mL) (mean)</td>
<td>2.37 × 10E6</td>
<td>3.61 × 10E6</td>
<td>NS</td>
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<td>HIV co-infection (N)</td>
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<td>Baseline ALT (U/mL) (median) (range)</td>
<td>81 (30–500)</td>
<td>59 (45–71)</td>
<td>NS</td>
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<td>Treatment-experienced (N, %) *</td>
<td>34 (69%)</td>
<td>3 (75%)</td>
<td>NS</td>
</tr>
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<td>Fibroscan (kPA, median) (range)</td>
<td>16.6 (9.5–66.4)</td>
<td>20.5 (11.1–28.0)</td>
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<tr>
<td>Cirrhosis (N, %)</td>
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<td>Child-Pugh Score (N, %)</td>
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<td>5</td>
<td>30 (61%)</td>
<td>3 (75%)</td>
<td>NS</td>
</tr>
<tr>
<td>6</td>
<td>25 (83%)</td>
<td>2 (67%)</td>
<td></td>
</tr>
<tr>
<td>Thrombocytes &lt; 90 (10E9/L) (N, %)</td>
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<td>1 (25%)</td>
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<tr>
<td>Thrombocytes &lt; 150 (10E9/L) (N, %)</td>
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<td>RVR (N, %)</td>
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<tr>
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<td>Hypertension</td>
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<tr>
<td>Dyslipidemia</td>
<td>22 (45%)</td>
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<tr>
<td>11 (22%)</td>
<td>1 (25%)</td>
<td></td>
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<tr>
<td>9 (18%)</td>
<td>2 (50%)</td>
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<tr>
<td>Decompensation of liver disease</td>
<td>0</td>
<td>0</td>
<td></td>
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</table>

* Treatment-experienced: earlier treatment with combination therapy with (peg)interferon and ribavirin; none of the patients were protease inhibitor (PI) experienced.

Figure 1. SVR12 in all patients, patients treated with or without RBV, patients with or without cirrhosis, treatment-experienced patients and patients with thrombocyte count < 90 x10E9/L. Abbreviations: TE: Treatment-experienced defined as earlier treatment with either classical interferon alone, or combination therapy with (peg)interferon and ribavirin; T < 90: Thrombocyte count < 90 x10E9/L.
DISCUSSION

In real-life 92% of HCV genotype 4 patients with advanced fibrosis/compensated cirrhosis treated with SMV and SOF for 12 weeks achieved SVR. Real-life data on treatment with SMV and SOF in HCV genotype 4 patients are limited. Our data confirm that treatment with a combination of SMV and SOF for 12 weeks is a good option in HCV genotype 4 patients with advanced fibrosis or compensated cirrhosis. In our cohort the four patients with virological failure did not receive RBV. Three of four failures were non-responders to previous interferon-based therapy, and three of four were cirrhotic patients. Although the number of failures was very low, the addition of RBV could be considered in patients with cirrhosis and non-response to previous interferon-based therapy. Earlier studies in HCV genotype 1 patients to assess treatment success of a 12-week treatment regimen of SMV and SOF showed SVR-rates of 83–86% in patients with advanced fibrosis/compensated cirrhosis. Recently, a real-world study showed similar SVR rates in HCV genotype 1 patients with cirrhosis treated with 12 weeks of SMV and SOF and no additional effectiveness of RBV. Whether the addition of RBV in cirrhotic patients leads to a higher SVR-rate in genotype 4 patients is not known. In our study cohort, all 12 patients who were treated with SMV, SOF and RBV achieved SVR whereas the four patients with a viral relapse did not receive RBV. Although this difference was not statistically significant, there might be a place for the use of RBV in treatment-experienced HCV genotype 4 patients with advanced fibrosis/cirrhosis. Findings from a recent study in cirrhotic genotype 1 CHC patients treated with SOF/SMV,
showed a lower SVR-rate of 79% in treatment-experienced patients compared to 88% in treatment-naïve patients.\textsuperscript{15} There were no differences in comorbidity and co-medication between the patients with and without SVR. Whether adherence to therapy may have been a factor remains the question, as we did not assess compliance in our study. A difference in SVR-rates between patients with HCV/HIV co-infection and patients with HCV mono-infection with genotype 4 could not be assessed in our study cohort as only a small number of patients (n = 4) had an HCV/HIV co-infection, of which all achieved SVR.

Limitations of our study are the relatively small number of included patients and the fact that the use of RBV was not randomised. As the addition of RBV was determined according to the discretion of the physician, there may be a bias towards RBV-use in more severe cirrhotic patients and/or treatment-experienced patients. As a result, a reliable comparison between SVR-rates in patients treated with or without RBV cannot be made.

Although there are currently more all-oral treatment options with DAA's available for genotype 4 HCV-infection (such as ledipasvir/sofosbuvir, daclatasvir/sofosbuvir or paritaprevir/ombitasvir/ritonavir/ ribavirin), it is worthwhile to have the combination of SMV and SOF (+/- RBV) as an efficacious treatment option. In various countries, not all of the mentioned combinations are registered or available and pricing upon which treatment regimen choices are made might differ.

In conclusion, in our real-life cohort we showed that combination therapy for 12 weeks with SMV and SOF in patients with HCV genotype 4 infection and advanced fibrosis/compensated cirrhosis was an effective regimen, with an overall SVR-rate of 92%. The effectiveness of adding RBV was unclear, but could be considered, as is recommended in recent AASLD and EASL guidelines for treatment-experienced and advanced cirrhosis patients.
REFERENCES


CHAPTER 6

The Observed Effect of Gastric Bypass Surgery on the Treatment of Chronic Hepatitis C Virus (HCV) Infection; A Case Report

E. Smolders, S. B. Willemse, O. El-Sherif, S. Khoo, D. Burger

In press Annals of Hepatology 2017
ABSTRACT

Chronic hepatitis C virus (HCV) infection can be cured with treatment using direct-acting antivirals (DAAs). Although these drugs have been widely studied, information about certain special populations are missing. In this case report we describe a treatment-experienced patient with chronic HCV infection genotype 1b, treated with 150mg/day simeprevir, 400mg/day sofosbuvir, and 1000mg/day ribavirin for 24 Weeks, after a Roux-and-Y gastric bypass. At steady-state a pharmacokinetic curve was recorded of sofosbuvir, GS-331007, and simeprevir. Ribavirin trough plasma concentration (C\text{trough}) was determined.

The simeprevir area under the time curve (AUC\text{last}) and C\text{trough} were 9.42 h·mg/L and 0.046mg/L, respectively. Compared to what was described in the literature, simeprevir exposure was low and therefore the simeprevir dose was increased to 300mg/day. The increased dose of simeprevir was well tolerated and C\text{trough} was 0.532mg/L. Sofosbuvir AUC\text{last} and C\text{trough} were 0.63 h·mg/L and 0.0013mg/L. GS-331007 AUC\text{last} and C\text{trough} were 21.02 h·mg/L and 0.35mg/L. Ribavirin C\text{trough} was 2.5mg/L. Sofosbuvir, GS-331007, and ribavirin exposure were comparable with levels described in literature. The patient achieved a sustained virologic response twelve weeks after the completion of treatment.
INTRODUCTION

Direct-acting antivirals (DAAs) are highly effective for the treatment of chronic hepatitis C virus (HCV) infection. Simeprevir is a second generation protease inhibitor (PI) which is a highly active DAA, especially in combination with sofosbuvir for the treatment of HCV-infection genotype 1 and 4.1,2 The relatively new DAAs have been widely studied but information about certain special populations is often not known. We are the first to describe a patient who underwent bariatric surgery and who, after relapsing to previous DAA therapy, was successfully treated for HCV-infection genotype 1b with sofosbuvir (Sovaldi®; Gilead Sciences, Cambridge, United Kingdom) simeprevir (Olysio®, Janssen-Cilag International, Beerse, Belgium), and ribavirin.

CASE REPORT

We describe a 61-year old Brazilian female patient, who presented to our outpatient clinic in 2011 with chronic HCV genotype 1b infection. She was diagnosed with HCV-infection in 2008, but the transmission route was unknown. Possible sources of infection included dental treatment or a caesarean section in Brazil.

The patient was severely obese with a body mass index (BMI) of 35.4 kg/m² (weight 84 kg, height 1.54 m). Ultrasound demonstrated hepatic steatosis without any ultrasonographic signs of cirrhosis. Evaluation of liver stiffness using Fibroscan®3,4 showed a value of 13.6 kPa, consistent with META VIR5,6 fibrosis score F3 (severe fibrosis). A liver biopsy showed moderately active periportal inflammation and moderate periportal fibrosis with formation of septae in less than 50% of portal fields (META VIR score A2/F2-3) and macrovesicular steatosis in 40–50% of hepatocytes with minimal pericellular fibrosis (Brunt score steatosis grade 2, fibrosis stage F1).7 Laboratory testing showed mildly elevated liver enzymes with an ALT of 77 U/L, AST of 51 U/L and gamma-GT of 57 U/L. Serum bilirubin, prothrombin time, albumin, creatinine, thrombocytes, and fasting blood glucose values were all normal. HCV RNA was 9.56 × 10⁵ IU/mL and HBsAg and anti-HIV 1 & 2 antibodies were negative.

The patient was a non-responder to treatment with peginterferon-alpha and ribavirin in 2009. In 2013, she was included in a clinical trial and was treated with DAAs daclatasvir and asunaprevir for 24 weeks. A relapse occurred after this treatment.

Whilst waiting for registration and reimbursement of the first DAAs in the Netherlands the patient decided to undergo gastric bypass surgery in 2014 (Roux-and-Y gastric bypass). She came back to our outpatient clinic in 2015 for (re-)treatment of the chronic HCV-infection. Her weight had reduced to 59 kg (BMI 24.9 kg/m²), and transaminases had improved (ALT 48 U/L; AST 39 U/L). All other liver enzymes and liver function tests were not altered and HCV RNA load was 5.64 × 10⁶ IU/mL. Sequencing of the viral genome was performed on the regions NS5A and NS3 (as she had received a NS5A inhibitor and a PI), which showed a high level of resistance associated substitutions (RAS) to NS5A inhibitors on the loci L31M/I and Y93H. There were no RAS present in the NS3 gene of the viral genome. For these reasons, we decided to treat the patient with 400mg sofosbuvir once daily, 150mg simeprevir once daily and 1000 ribavirin per day, for a total of 24 weeks.8–10
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Figure 1. Plasma concentrations of 400mg sofosbuvir and GS-331007 (A) and 150 and 300mg simeprevir (B).

A: Sofosbuvir concentrations at Week 3: C\text{trough}: 0.001 mg/L; C\text{max}: 0.35 mg/L; T\text{max}: 2.25h; AUC\text{0-24}: 0.63 h*mg/L; T\text{1/2}: 0.5h. GS-331007: C\text{trough}: 0.35 mg/L; C\text{max}: 1.55 mg/L; T\text{max}: 4.91h; AUC\text{0-24}: 21.02 h*mg/L; T\text{1/2}: 10.3h. Sofosbuvir reference values (400 mg once daily treatment-naïve HCV genotype 1-infected subjects without cirrhosis). Sofosbuvir: T\text{max}: 0.5-1.5h; C\text{max}: 0.55mg/L; AUC\text{0-24}: 1.03 h*mg/L. GS-331007: T\text{max}: 2-4h; C\text{max}: 582 mg/L; AUC\text{0-24}: 7.12 h*mg/L.

B: Simeprevir concentrations at Week 3: C\text{trough}: 0.046 mg/L; C\text{max}: 1.20 mg/L; T\text{max}: 3.25h; AUC\text{0-24}: 9.41 mg/L; T\text{1/2}: 4.6h. Week 14: C\text{trough}: 0.532 mg/L. Simeprevir reference values (treatment experienced patients 150mg, once daily): C\text{trough}: 1.41 mg/L; C\text{max}: 4.38 mg/L; T\text{max}: 2.03-9.87h; AUC\text{0-24}: 57.4 h*mg/L.
The effect of gastric bypass surgery on the absorption of the DAAs is unknown. Simeprevir and ribavirin in particular must be taken with food for adequate plasma concentrations. However, due to the bariatric surgery, the patient was not able to eat large meals. To study the exposure of the DAAs and ribavirin in this patient, a pharmacokinetic curve was obtained at Week 3 of DAA treatment. Blood was sampled at t = 0 (pre-dose), 2, 3, 5, 6, 8, and 24 hours after intake of the DAAs. DAA plasma concentrations were determined using an in-house made, validated HPLC-MS/MS tandem mass spectrometry assay and used to calculate pharmacokinetic parameters. The assay lower limits of quantification for sofosbuvir, GS-331007 and daclatasvir were 2.5ng/mL, 10ng/mL, and 10 mg/L respectively. The precision for low, medium and high quality control (QCs) samples was <10 % for all analytes. Ribavirin plasma concentrations were determined using validated HPLC assay with UV detection.

At Week 3, the area under the time curve (AUClast) for sofosbuvir was 0.63 h.mg/L, the maximum plasma concentration (Cmax) was 0.35 mg/L, and the minimum plasma concentration (Ctrough) was 0.0013 mg/L. For the main inactive metabolite of sofosbuvir, GS-331007, the AUC0-24 was 21.02 h.mg/L, Cmax was 1.55 mg/L, and the Ctrough was 0.35 mg/L (Figure 1a).

For simeprevir, at Week 3 of treatment, the AUC0-24 was 9.42 h.mg/L, the Cmax was 1.21 mg/L, and the Ctrough was 0.046 mg/L (Figure 1b). Ribavirin concentration was 2.5mg/L. Sofosbuvir and ribavirin concentrations were considered adequate but simeprevir concentrations were sub-therapeutic compared with those described in literature. As a result, at Week 10 of treatment, the simeprevir dose was doubled to 150 mg twice daily (taken together with food). At Week 14 the trough concentrations of ribavirin and simeprevir were determined again and the Ctrough of simeprevir and ribavirin were 0.532mg/L and 3.5mg/L, respectively. The haemoglobin level had dropped from 12.3 g/dL to 9.8 g/dL. HCV RNA was undetectable during treatment at Week 3, 4, 12, 24 (end of treatment) and 12 weeks after end of treatment (sustained virologic response, SVR12).

During treatment, the main side effect was extreme fatigue. Liver enzymes, liver function tests and renal function were all normal during treatment.

For this case report no formal ethical approval was obtained as all procedures were performed for regular health care purposes. The patient did not have to comply to certain extra examinations of life style rules. However, the patient gave consent for performing the pharmacokinetic curve and publication of this paper. This was recorded in the patient chart.

**DISCUSSION**

We are the first to describe a patient who was successfully treated with DAAs including an adjusted dose of simeprevir after undergoing gastric bypass surgery. Although simeprevir was not deemed to be ideal in this patient, given the food-dependent uptake, there was no alternative choice due to existing resistance to NS5A inhibitors.

The goal of bariatric surgery is to decrease the intake of food and absorption of nutrients for severely obese patients, resulting in weight loss. These surgeries also affect drug absorption of orally administered drugs, as the gastrointestinal (GI) tract is substantially altered. Little is known about the effects of a Roux-and-Y gastric bypass on drug exposure.
On one hand, gastric pH rises which could cause increased absorption. On the other hand, absorption could decrease, as the transit time of a drug through the GI tract is reduced.\textsuperscript{16}

We treated the patient for 24 weeks, according to national and international guidelines, as she relapsed to earlier dual NS3/NS5A DAA therapy.\textsuperscript{8-10} We also tried to enhance the potency of the treatment by adding ribavirin (at a weight-based dose). Simeprevir and ribavirin are both recommended to be taken with food, as food intake increases the absorption of both drugs.\textsuperscript{11,17} According to the simeprevir label, the AUC increases by approximately 60\% when administered with a fatty meal or normal breakfast.\textsuperscript{11} In this case, Simeprevir $C_{\text{trough}}$ levels were 97\% lower than comparable reference values, and the $AUC_{0-24}$ was 84\% lower. Our patient was not able to have large or “normal sized” meals (i.e. a high intake of calories) anymore and we postulate that this resulted in the extremely low exposure to simeprevir. Despite the fact that HCV RNA was undetectable, we doubled the dose of simeprevir to increase the plasma exposure and efficacy. This dose was well-tolerated and the $C_{\text{trough}}$ Plasma concentration at Week 14, (4 Weeks after doubling the dose), was approximately 11-fold higher than the Week 3 $C_{\text{trough}}$ level (62\% lower than the reference value). This extreme increase is the result of the non-linear pharmacokinetics of simeprevir.

For ribavirin, we strived to attain a plasma concentration of 2.0-3.0 mg/L at steady-state.\textsuperscript{18} At Week 3 of treatment the plasma concentration was already 2.5 mg/L, which is remarkable as the patient had a low intake of food.\textsuperscript{17} These high ribavirin levels caused anaemia and the patient suffered from extreme fatigue. It was considered to lower the dose of ribavirin, but because the haemoglobin levels remained stable throughout the whole course of treatment and the patient did not want a dose reduction, the starting dose of 1000mg/day was continued. The high plasma concentrations of ribavirin (compared to the low levels of simeprevir) could also be related to the low body weight of < 60 kg of the patient after gastric bypass surgery. The fact that a large or “normal” meal could not be consumed seems less important for an adequate ribavirin level as the initial dose was already relatively high.

Sofosbuvir pharmacokinetics were not affected by the gastric bypass or the low intake of food as the exposure to both sofosbuvir and GS-331007 (the main inactive metabolite of sofosbuvir) were sufficient. This was as expected because it was earlier described that a high-fat meal does not influence the plasma concentration of sofosbuvir or GS-331007.\textsuperscript{19}

This case report describes a patient with chronic HCV-infection genotype 1b without liver cirrhosis, but with a relapse after earlier dual DAA-treatment, who was successfully treated with simeprevir, sofosbuvir, and ribavirin for 24 weeks after undergoing gastric bypass surgery. Adequate sofosbuvir and ribavirin plasma concentrations were achieved, however, simeprevir plasma concentrations were low when simeprevir was dosed according to the drug label (150 mg once daily).\textsuperscript{11} Both bariatric surgery and low intake of food can influence drug absorption and drug exposure. Awareness is needed when patients who underwent bariatric surgery are treated with certain drugs without any experience in this specific condition. This is especially the case for simeprevir, as absorption is dependent of food intake, it has non-linear pharmacokinetics and possibly more severe side effects when given in high doses. Patients with a history of bariatric surgery who are treated with simeprevir should be closely monitored using, for example, therapeutic drug monitoring.
ACKNOWLEDGEMENTS

We thank the patient for participating. Secondly we would like to thank the laboratory personnel at the Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom and the laboratory personnel at the Clinical Pharmacy of Radboud university medical center, Nijmegen. We also thank the nurses and pharmacy personnel Academic Medical Center, Amsterdam, the Netherlands, for their support during the hospital admission of the patient. We thank Yuma Bijleveld for her support during the hospital admission.
REFERENCES


CHAPTER 7

The Estimated Future Disease Burden of Hepatitis C Virus in the Netherlands with Different Treatment Paradigms


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ABSTRACT

Background & Aims
Prevalence of hepatitis C virus (HCV) infection in the Netherlands is low (anti-HCV prevalence 0.22 %). All-oral treatment with direct acting antivirals (DAA’s) is tolerable and effective but expensive. Our analysis projected the future HCV-related disease burden by applying different treatment scenarios in the Netherlands.

Methods
Using a modeling approach, the size of the HCV-viremic population in the Netherlands in 2014 was estimated using available data and expert consensus. The base scenario (based on the current Dutch situation) and different treatment scenarios (with increased efficacy, treatment uptake, and diagnoses) were modelled and the future HCV disease burden was predicted for each scenario.

Results
The estimated number of individuals with viremic HCV infection in the Netherlands in 2014 was 19,200 (prevalence 0.12 %). By 2030, this number is projected to decrease by 45 % in the base scenario and by 85 % if the number of treated patients is increased. Furthermore, the number of individuals with hepatocellular carcinoma and liver-related deaths are estimated to decrease by 19 % and 27 % respectively in the base scenario, but may both be further decreased by 68 % when focusing on treatment of HCV-patients with a fibrosis stage of ≥ F2.

Conclusions
A substantial reduction in HCV-related disease burden is possible with increases in treatment uptake as the efficacy of current therapies is high. Further reduction of HCV-related disease burden may be achieved through increases in diagnosis and preventative measures. These results might inform the further development of effective disease management strategies in the Netherlands.
INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major cause of chronic liver disease. It causes liver fibrosis and may ultimately lead to liver cirrhosis, hepatocellular carcinoma and death. It has been estimated that there are around 80 million people worldwide with chronic HCV infection. There is a large geographical variation in prevalence of HCV-infection and in many countries the epidemiology of HCV-infection is not well known. At the same time, HCV-related mortality continues to increase as the infected population ages and the infected population advances to late-stage liver disease.

Recently, the World Health Organization (WHO) recognized viral hepatitis as a global public health problem, and asked countries to develop comprehensive national viral hepatitis strategies. In The Netherlands, estimates on antibody prevalence of HCV-infection vary from 0.1 to 0.6 %. The most recent and reliable nationwide estimate was 0.22 % (0.07 %–0.37 %) in Dutch habitants aged 15–79 years in 2009, incorporating prevalence data among different subpopulations, corresponding with about 28,000 adult individuals ever infected with HCV. Assuming a spontaneous clearance rate of 26 %, around 20,000 of them are or have been viremic. This corresponds to a viremic prevalence of 0.13 % in the total Dutch population. The risk groups of individuals with a known viremic HCV-infection (relatively many (ex-) drug users) are different from the groups of individuals currently at risk of a new HCV-infection (strikingly almost no drug-users, but mainly HIV-positive men who have sex with men). This situation is different in The Netherlands compared to many other countries where HCV transmission among people who inject drugs (PWID) is ongoing. Importantly, the undiagnosed population might be substantial due to the symptom-free course in approximately 80 % of cases. A study from the southern region of The Netherlands indicated that 66 % of HCV-infections is hidden to current screening practices (“hidden population”).

With the availability of new powerful peginterferon-free treatment modalities in sight, treatment of HCV will be more effective and has fewer side effects. As a result, the barriers for starting treatment are expected to be lower and as a result more patients will be (successfully) treated. Following the recommendations of the WHO, it is important to develop a strategy to diagnose the “hidden” HCV-infected population in The Netherlands in order to be able to benefit from the treatment advances. However, reliable data on epidemiology and understanding of disease dynamics and barriers to HCV screening and treatment are needed before robust plans can be made.

The aim of this study was two-fold: The first aim was to estimate the future disease burden for The Netherlands using available data and expert opinion if the current treatment paradigm and cure would be continued.

The second aim was to show the impact of different intervention strategies on the future disease burden. Extreme strategies were considered to illustrate the potential range of outcomes. The reality may fall within one of these strategies. The focus of this analysis was not prescriptive, stating what should be done to reduce HCV-infection disease burden. Instead, the focus was descriptive, showing the impact on disease burden if certain assumptions can be met. Cost-effectiveness analyses were not considered. This study is part of a larger project to quantify HCV-epidemiology in countries around the world in a systematic manner, and for which the same prediction model has been used. In our report, we focus on the situation in The Netherlands.
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METHODS

Baseline population characteristics

Inputs
A systematic review of the literature was conducted to identify studies reporting the total number of HCV cases diagnosed, treated and cured in the Netherlands. Indexed articles were found by searching PubMed and Embase. The review encompassed all studies between January 1990 and July 2013. Non-indexed sources were identified through ministry of health websites and international agencies’ reports. As described in detail in an earlier published study, this literature search was combined with face-to-face discussions with a panel of experts (consisting of epidemiologists, hepatologists, infectious disease specialists, public health professionals and virologists) to gather epidemiological data and consensus estimates. The obtained data were used to estimate the historical number of new HCV-infections per calendar year.

Model
A disease progression model was constructed in Microsoft Excel® (Microsoft Corp., Redmond, WA) to quantify the size of the HCV-infected population, by the liver disease stages (META VIR score F0-F4), from 1950–2030. The model was set up for sensitivity and Monte Carlo analysis using Crystal Ball®, an Excel® add-in by Oracle®. Beta-PERT distributions were used for all uncertain inputs. The Excel® optimization add-in, Frontline Systems’ Solver, was used to calculate the number, age and gender distribution of the annual acute infections. The model was validated in countries where annual HCC incidence and liver-related deaths were reported. The model was used to calculate the number, age and gender distribution of the annual acute HCV-infections, that progressed to chronic HCV-infection after accounting for spontaneous clearance of the virus (Figure 1). The progression of these new cases was followed along with all chronic infections from prior years. Unless specified, the scope of the model was limited to HCV-viremic (ribonucleic acid (RNA) positive) cases. Non-HCV-viremic cases (those who spontaneously cleared the virus or were treated and cured) were not considered even though they would test positive to HCV antibodies and may still progress to more advanced stages of liver disease despite viral clearance. In addition, re-infections following spontaneously or treatment-induced clearance were not considered as it was not possible to add this factor to the prediction model we used. The total number of cases, at each stage of the disease, was tracked by age and gender. Five-year age cohorts were used through age 84; those aged 85 and older were treated as one cohort. Each year, one fifth of the population in each age group, except for 85 and older, was moved to the next age cohort to simulate aging.

Estimation of chronic and new HCV-infections

Prevalence of HCV-infections
Available data were used to estimate the number of adults living with an HCV-RNA positive infection in The Netherlands. The paper we used for estimating anti-HCV-antibody prevalence was chosen because it was the most recent estimate and had the
best representation of the overall population in the Netherlands. There were no reliable age and gender distributions available for The Netherlands but the median age was reported at 54 years in 2006-2007, slightly younger than in the United States. In addition, United States and Dutch gender ratios were considered comparable, as well as the timing of the peak infections, so the Dutch age and gender distributions were established using the United States as an analog (Figure 2). Dutch population data were obtained by 5 year age and gender cohorts from the United Nations population database, which uses the data registered at the Dutch central bureau for statistics (Statnet). The genotype distribution (Table 1) was established using data from an analysis of patient data collected between 2002 and 2005 from 53 hospitals in 11 of the 12 Dutch provinces.

**Diagnosed HCV infections**
The annual number of newly diagnosed HCV cases ranged from 400 to 800 according to the expert panel. This range was based on different recent and less recent reports. One of these data sources is the compulsory reporting system for new HCV-infections from 1999 to 2003, in which 600-700 new infections were reported per year (3.9-4.1 per 100,000 inhabitants). Another data source is the information system of Dutch microbiology laboratories reporting the number of positive HCV tests per year. Not all laboratories participate, giving an under-estimation, but there are also patients tested more than once per year, which may compensate for this under-estimation. From 2005 to 2010 there were 700 to 900 diagnoses per year, and from 2011-2014 the number declined to 380 diagnoses per year. By 2013, it was estimated that 12,000 individuals were diagnosed (an average of 600 newly diagnosed cases per year over 20 years). In 2013, based on estimations of the expert panel in combination with data in the literature, it was estimated that 650 individuals were newly diagnosed with HCV viremia.

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**Figure 1.** The flow of the HCV disease progression model
New HCV infections

The annual number of new cases (i.e. acute HCV-infections and new chronic HCV-infections due to immigration) did not remain stable since 1950. Thus, an annual relative incidence value was used to describe the change in the number of new infections over time. Relative incidence was set to 1 in 1950, and based on discussion with the expert panel, taking into account the risk factors common in The Netherlands over time (nosocomial infections before 1992, injection drug use, etc.), it was estimated that the number of new infections peaked in 1989 and gradually declined thereafter. In 2013, 62 new cases of acute HCV-infection were notified to the National Institute of Public Health and the Environment (RIVM). Of these cases only two were notified to be due to injecting drug use (IDU) and an earlier study performed in 1999-2001 showed that 6% of all new HCV cases were attributable to IDU. In line with these findings, cohort studies show a very low incidence. Therefore, in the model the annual number of new cases due to IDU was considered low. In The Netherlands, like in many other countries, transfusion of blood products is considered no longer a risk factor for new HCV-infections since 1992, as donor blood screening started in 1991. A linear declining rate was applied to get the percentage of total infections attributed to transfusion to zero by 2030. The annual number of new cases due to immigration was calculated by gathering net annual immigration, by country of origin and the corresponding anti-HCV prevalence in the country of origin. Based on the immigration data the numbers increase from 1995 until 2011, and then stay constant 2011 onwards. Another group with high risk of a new HCV-infection is the group of HIV-positive men who have sex with men (MSM). Of the 362 newly reported HCV-infections in 2013, 155 were among HIV-positive MSM. The risk of re-infection is considered low among PWID but substantial among MSM in The Netherlands.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>14.8</td>
</tr>
<tr>
<td>1b</td>
<td>15.7</td>
</tr>
<tr>
<td>1 Other/NA</td>
<td>18.8</td>
</tr>
<tr>
<td>2</td>
<td>9.7</td>
</tr>
<tr>
<td>3</td>
<td>19.1</td>
</tr>
<tr>
<td>4</td>
<td>10.5</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
</tr>
<tr>
<td>6</td>
<td>0.0</td>
</tr>
<tr>
<td>Other</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Netherlands. However, in the model we used, it was not possible to consider re-infections. The model calculated the annual number of all-cause and liver-related deaths, and the cured cases as described below:

**Progression Rates**

Disease progression by age and sex was simulated by multiplying the total number of cases at a particular stage of the disease by a progression rate to the next stage. The rates were gathered from previous studies or calculated using known numbers from Dutch national reports. Liver transplant data were available through the Eurotransplant Statistics Report Library and from the individual transplant centers in The Netherlands. In 2013, there were 142 liver transplants performed in The Netherlands. Of all liver transplants 12% are attributable to HCV-infection each year (a frequency of 11–13% over the past 12 years, based on personal communication with the three transplant centers in The Netherlands). The total number of cases was adjusted for aging, all-cause mortality and proportion of cured HCV infections in any given year.

**All-Cause Mortality**

The all-cause mortality rates by age and gender were gathered from the Human Mortality Database. Mortality rates were adjusted using standard mortality ratios among PWID and individuals having received blood products.

**Treated & Cured**

Analysis of ribavirin (RBV) units sold (for chronic or acute HCV-infection) were used to estimate the total number of treated HCV patients in The Netherlands. In 2013, this number was 880. It was assumed that the number of treated patients stayed constant after this last reported year (2013). It was also assumed that the number of treated patients for each genotype was proportional to the genotype distribution of the HCV-infected population. The annual number of cured patients was estimated using the average sustained viral response (SVR) rate of the different treatments in a given year (SVR-rates were based on available literature). A separate SVR was used for the major genotypes, as shown in Figure 3. A weighted average of different treatment options in a given year was considered (dual therapy with peg-IFN and RBV or triple therapy with peg-IFN, RBV and a direct-acting antiviral (DAA)). The number of cured patients from all genotypes was summed by stage of the disease and we assumed that the numbers were equally distributed among eligible age cohorts.

**Treatment protocols and strategies**

The model interface allowed for changing assumptions for the number of patients treated, the proportion of cases eligible for treatment, the reduction in treatment restrictions, the average sustained viral response (SVR) rate by genotype, the number of newly diagnosed individuals and the number of new infections at five different points in time. The year in which these changes were observed was also an input field. Different new therapies considered were: DAA + peg-IFN + RBV, DAA + RBV (interferon-free) and all-oral DAA combinations with or without RBV. For the model, we assumed that all changes took effect immediately. The coexistence of multiple therapies was handled by modifying the average SVR.
Increased efficacy and treatment uptake

Figure 3. Model inputs for the "Increased efficacy and treatment scenario", by calendar year.
The pool of patients who could be treated was impacted by explicit or implicit treatment protocols. According to the literature, approximately 40–60% of HCV patients are eligible for peg-IFN/RBV.\(^{43-45}\) The definition of eligibility includes contra-indications to the drugs as well as patient’s preference. In this analysis, 60% of the patients were considered treatment eligible for standard-of-care (Figure 3.), being peg-IFN plus RBV for genotype 2 to 6 and peg-IFN plus RBV plus DAA for genotype 1. When peg-IFN could be eliminated, the eligibility was increased. We assumed that the increase in eligibility did not directly increase the number of patients treated in the future. However, we assumed that it did increase the pool of diagnosed and eligible patients who could be drawn upon. Any changes in treatment were implemented using a separate input.

The future number of treated patients was capped by (I) the number already diagnosed, (II) number eligible and (III) unrestricted cases. The latter related to implicit (defined by physicians’ practice) and/or explicit (defined by treatment guidelines) restrictions. These restrictions could be modified by changing the upper and lower end of patients’ age and their stage of fibrosis (F4 (Child-Pugh A, B or C), F3, F2, or F1/F0). Review of treatment guidelines and interviews with the expert panel were used to identify both of these factors. Decompensated cirrhotic patients were considered ineligible for peg-IFN-containing therapies (irrespective of genotype). When the number of treated patients was greater than those diagnosed, eligible and unrestricted, the number of newly diagnosed cases was increased or the treatment restrictions were loosened. The focus of the analysis was to highlight how many cases have to be diagnosed to achieve a treatment strategy rather than to forecast the screening capacity.

**Scenarios**

Multiple treatment strategies were considered and are described below: base scenario, increased efficacy only, increased efficacy and treatment uptake, screening and elimination, and focused treatment of individuals with different fibrosis stages. Scenario inputs, including SVR, fibrosis stage and medical eligibility, divided by genotype and year, are shown in Figure 3. The numbers of treated and diagnosed patients necessary to achieve the desired scenario outputs are also shown.

In all instances, HCV-viremic infections represented all current HCV-infections (acute and chronic HCV-infections). The term viremic was used throughout this study to highlight the presence of HCV-RNA. The term incidence was used for new HCV infections per calendar year and not newly diagnosed. HCC referred to the total number of viremic HCV-related HCC cases, rather than new cases. Additionally, all reductions by disease stage were assumed to occur among the viremic HCV-infected population. The effects of non-HCV-related liver disease were not considered in this analysis.

**Base scenario**

The base scenario was defined as the scenario where all assumptions (the number of acute cases, treated patients, percentage of patients eligible for treatment, treatment restrictions, the number of newly diagnosed and the average SVR by genotype) remained the same as in 2013-2014. The base scenario was previously described in detail, together with other countries.\(^{17,46}\) and was assumed to be the most conservative scenario. Even more conservative scenarios are possible (e.g., stop treating HCV-infected patients completely), but those were deemed to be unlikely.
As described above, we assumed in this scenario 650 newly diagnosed HCV infections annually and treatment of 880 HCV infections annually in The Netherlands. Treatment in this scenario was focused on patients of 15-69 years of age and with a METAVIR score of ≥ F3 assessed using FibroScan. In the light of a future high treatment rate, we considered patients with a fibrosis stage of ≥ F2 (according to METAVIR, measured using FibroScan) eligible for treatment in 2018, and patients with a fibrosis stage of ≥ F0/F1 eligible for treatment in 2021. We assumed SVR rates of 70% for genotype (G) 1 and G3, 80% for genotype 2 (G2) and 50% for genotype 4 (G4). We used fibrosis scores obtained using FibroScan because that is the most common mode of fibrosis assessment at the moment.

**Increased efficacy only**

A second scenario was developed to assess the impact of improved treatment efficacy without changes in the number of treated or diagnosed patients. Treatment age and fibrosis staging eligible for treatment as presented in the base scenario was held constant. In 2015, it was projected that SVR could increase to 80% for G1 and G4, 90% for G2 and 75% for G3. In 2016, SVR was estimated 90% across all genotypes. These rates were held constant through 2030.

**Increased efficacy and treatment uptake**

A third scenario was created to assess the actions necessary to eliminate chronic HCV-infection by 2030. Beginning in 2015, treatment uptake was increased with 10% across all genotypes to 970 individuals and the number of diagnoses was increased with 25% to 810 individuals annually. Treatment was open to individuals 15-69 years of age. In 2016, treatment uptake was increased to 1,210 individuals annually and diagnosis was increased to 890 individuals annually. Patients with fibrosis ≥F2 were considered eligible for treatment. In 2018, treatment uptake was increased to 1,700 individuals annually. Treatment was now also open for patients with fibrosis >F0/F1 and the eligible age range was increased up to 74 years. Treatment and diagnosis uptake were held constant from 2018 through 2030. In 2021, all patients, regardless of fibrosis staging, were eligible for treatment.

**Screening and elimination**

A fourth scenario was created to assess the response of increased treatment and the corresponding required increase in screening (and diagnoses) to keep up with treatment. In addition, it was assumed that preventive measures will be taken to reduce the number of new infections by 40% over six years.

**Focused treatment: ≥F3, ≥F2, ≥F0/F1**

A fifth, sixth and seventh scenario were created to assess the impact of focused treatment of individuals with fibrosis ≥F3, ≥F2 and ≥F0/F1. Starting in 2015 treatment uptake was increased by 10% across all genotypes to 970 individuals and the number of diagnoses was increased to 25% to 810 individuals. In 2016, the treatment uptake increased 25% to 1,210 individuals and the diagnosis rate increased 10% to 890 individuals annually. By 2018, the eligible age range was increased to 74 years while treatment was increased by 40% to 700 individuals as in this year treatment exceeded eligible individuals. For the ≥F2 and ≥F0/F1 scenarios treatment uptake was increased by 40% to 1,700 individuals. In 2021, the number of diagnoses was kept constant at 890
individuals. For the ≥F3 scenario 400 individuals were treated annually. For the ≥F2 scenario 530 individuals were treated as the treatment outpaced eligibility in 2020. For the ≥F0/F1 scenario 1,700 individuals were treated annually.

**Birth Cohort Effect**
The age distribution was determined as described above. The disease progression model was used to age the HCV-infected population after taking into account mortality and SVR. For this analysis, the median age in each five-year age cohort was selected and converted to a birth year. A range of birth years were selected which accounted for approximately 75% (or more) of the total HCV-infected population using the 2014 HCV-population distribution. The number of people that need to be screened to identify one viremic case was calculated by taking the inverse of the viremic HCV-prevalence. The number needed to screen to identify one new case was calculated as follows:

\[
1 \div (HCV \text{ Viremic Prevalence} \times (1 - \% \text{ of HCV Population Already Diagnosed}))
\]

**RESULTS**
The results of the literature review and expert opinion, including estimates of HCV-antibody and HCV-viremia prevalence, diagnosis, as well as annual treatment and liver transplants are summarised in Table 2.

**Base scenario**
Using historical data, it was estimated that there are around 19,200 individuals in The Netherlands with a viremic HCV infection in 2014. It was forecasted to decrease to 10,599 (45%) in 2030. The number of HCV-related HCC cases in 2014 was estimated at 110, and it was forecasted to decrease by 19% by 2030. The number of liver-related deaths in chronic HCV patients was forecasted to decrease 27% from a base of 102. Figure 2. shows the age and gender distribution of the HCV-infected population in 2009 while Figure 4. shows the projected age distribution in 2014. Figure 5. shows the number of viremic HCV infections over time in The Netherlands from 1950 to 2030 and Figure 6. shows the projected HCV disease burden for this period.

**Other Scenarios**
The results of the analyses for HCV morbidity and mortality, by scenario, are summarised in Figure 7. and the percent change from the base scenario can be found in Figure 8. Table 3. compares the change in HCV disease burden in 2014–2030 by scenario.

**Increased efficacy only**
There will be 2,413 fewer HCV-viremic individuals in 2030, a 23% reduction as compared to the base scenario. The number of HCV-related HCC cases and the number of liver-related deaths both decrease with 25% from the base scenario. This scenario would result in 463 cirrhosis cases being averted.
## Table 2. Model inputs and 2014 estimations

<table>
<thead>
<tr>
<th></th>
<th>Historical (min-max uncertainty interval)</th>
<th>Year (Reference)</th>
<th>2014 (95% uncertainty interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCV-Infected Cases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Anti-HCV Cases</td>
<td>29,450 (9,400-49,530)$^1$</td>
<td>2009</td>
<td>26,010 (7,140-45,820)</td>
</tr>
<tr>
<td>Anti-HCV Prevalence</td>
<td>0.2% (0.1%-0.3%)$^1$</td>
<td></td>
<td>0.2% (0.0%-0.3%)</td>
</tr>
<tr>
<td>Number of Viremic Cases</td>
<td>21,800 (6,370-36,650)$^3$</td>
<td>2009</td>
<td>19,200 (4,740-35,480)$^2$</td>
</tr>
<tr>
<td>Viremic Prevalence</td>
<td>0.13% (0.0%-0.2%)$^3$</td>
<td></td>
<td>0.12% (0.0%-0.2%)</td>
</tr>
<tr>
<td>Viremic Rate</td>
<td>74.0%$^3$</td>
<td></td>
<td>74.0%</td>
</tr>
<tr>
<td><strong>HCV Diagnosed (Viremic)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viremic Diagnosed</td>
<td>10,470$^1$</td>
<td>2013</td>
<td>10,200</td>
</tr>
<tr>
<td>Viremic Diagnosis Rate</td>
<td></td>
<td></td>
<td>51.1%</td>
</tr>
<tr>
<td>Annual Newly Diagnosed</td>
<td>650$^5$</td>
<td>2013</td>
<td>650</td>
</tr>
<tr>
<td><strong>New Infections</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Infections</td>
<td></td>
<td></td>
<td>510$^6$</td>
</tr>
<tr>
<td>New Infection Rate (per 100K)</td>
<td></td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td><strong>Treated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number Treated</td>
<td></td>
<td></td>
<td>880$^7$</td>
</tr>
<tr>
<td>Annual Treatment Rate</td>
<td></td>
<td></td>
<td>4.5%</td>
</tr>
<tr>
<td><strong>Risk Factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected via IDU (%)</td>
<td></td>
<td></td>
<td>4090 (21.3%)$^8$</td>
</tr>
<tr>
<td>Infected via blood transfusion (%)</td>
<td></td>
<td></td>
<td>6.0%$^3$</td>
</tr>
</tbody>
</table>

$^1$The reported prevalence of anti-HCV antibodies was 0.22% (28,100) in 15-79 year olds.$^8$ Prevalence in older and younger individuals was extrapolated resulting in an overall prevalence of 0.18% or 29,450 for all ages. The viremic rate was applied get both the viremic prevalence as well as the number of viremic cases.$^2$ Model estimate after considering new infections and cured.$^3$ Thomas et al$^{15}$. $^4$ Panel expert estimate – over the last 20 years, on average 600 individuals were newly diagnosed per year, the panel estimated 650 in 2013.$^5$ According to laboratory reports, 679 cases were newly diagnosed in 2011 and 500 cases in 2012. In 2013, it was estimated that 650 HCV infections were newly diagnosed (expert panel together with literature findings). $^6$ New viremic infections estimated using the following data: 6 among IDU, 354 among immigrants (used Statistics Netherlands to calculate net immigrants in 2011 (n=35,131) with an average prevalence of 1.01%; the average prevalence included an adjustment for a lower HCV prevalence among younger immigrants), 155 among HIV+ MSM (incidence rate of 12/1000$^{27}$ applied to 12,880 HIV+ MSM who are HCV negative of a total of 14,000 HIV+ MSM of whom 8% is already HIV/HCV co-infected), 6 nosocomial infections (range 4-8). This adds up to a total of 521 new infections.$^7$ GIP databank$^{41,42}$. $^8$ EMCDDA – European Drug Report 2013.$$^8$ Chaves et al$^{24}$. 
Figure 4. Distribution of HCV-infected population by birth year cohort 2014

Figure 5. The number of viremic HCV infections over time, The Netherlands 1950-2030 (base scenario)

Figure 6. HCV disease burden over time, The Netherlands 1950-2030 (base scenario). Decompensated cirrhosis figures excluded those who received a liver transplant.
**Increased efficacy and treatment uptake**
With an aggressive treatment and diagnosis strategy, there will be 9,043 fewer HCV-viremic individuals in 2030, an 85% reduction as compared to the base scenario. The number of HCV-related HCC cases and the number of liver-related deaths in 2030 decrease with 67% and 65% respectively from the base scenario. This scenario would result in 964 cirrhotic cases being averted.

**Screening and elimination**
With a screening and elimination strategy, there will be 9,334 fewer HCV-viremic individuals in 2030, an 88% reduction as compared to the base scenario. The number of HCV-related HCC cases and the number of liver-related deaths in 2030 decrease with 68% and 66% respectively from the base scenario. This scenario would result in 972 cirrhotic cases being averted.

**Focused treatment: ≥ F3**
There will be 1,610 more HCV-viremic individuals in 2030, a 15% increase as compared to the base scenario. The number of HCV-related HCC cases and the number of liver-related deaths in 2030 decrease with 57% and 60% respectively from the base scenario. This scenario would result in 825 cirrhotic cases being averted.

**Focused treatment: ≥ F2**
There will be 811 fewer HCV-viremic individuals in 2030, an 8% reduction as compared to the base scenario. The number of HCV-related HCC cases and the number of liver-related deaths in 2030 both decrease with 68% from the base scenario. This scenario would result in 965 cirrhotic cases being averted.

**Focused treatment: ≥ F0/F1**
There will be 8,999 fewer HCV-viremic individuals in 2030, an 85% reduction as compared to the base scenario. The number of HCV-related HCC cases and the number of liver-related deaths in 2030 decrease with 63% and 60% respectively from the base scenario. This scenario would result in 921 cirrhotic cases being averted.

**Birth Cohort**
The median age of the viremic HCV-infected population in 2014 was 51 years (birth year 1963). More than 50% of the viremic HCV-infected population was born between 1955–1969; over 80% were born between 1950–1979 (Figure 4). The highest prevalence of HCV-viremia is in the population born between 1960–1964 (0.31%). By focusing screening on this birth cohort it is estimated that one case can be newly diagnosed for every 659 screened (after taking into consideration those already diagnosed), if participation rates are equal among HCV-infected and -uninfected within this age cohort (Table 4). It was assumed that 51% of the total HCV-viremic population has been diagnosed for all age groups.
Figure 7. HCV characteristics, by scenario, The Netherlands 2013-2030
Clinical Studies on Hepatitis B, C, and E Virus Infection

Figure 8. Percent change from the base scenario to 2030 with treatment by scenario. Abbreviations: LRD: Liver Related Death, HCC: Hepatocellular Carcinoma

Table 3. Predicted number of viremic HCV-infections, cases with Hepatocellular carcinoma and liver-related deaths according to scenario from 2014 to 2030

<table>
<thead>
<tr>
<th>Scenario</th>
<th>HCV-Infections N</th>
<th>HCC N</th>
<th>LRD N</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014 (base)</td>
<td>19,200</td>
<td>110</td>
<td>102</td>
</tr>
<tr>
<td>Base scenario N (% decrease*)</td>
<td>10,599 (45)</td>
<td>89 (19)</td>
<td>74 (27)</td>
</tr>
<tr>
<td>Increased efficacy only N (% decrease*)</td>
<td>8,187 (57)</td>
<td>66 (40)</td>
<td>54 (47)</td>
</tr>
<tr>
<td>Compared to base scenario</td>
<td>- 23%</td>
<td>- 25%</td>
<td>- 25%</td>
</tr>
<tr>
<td>Increased efficacy and treatment uptake N (% decrease*)</td>
<td>1,556 (92)</td>
<td>30 (73)</td>
<td>25 (75)</td>
</tr>
<tr>
<td>Compared to base scenario</td>
<td>- 85%</td>
<td>- 67%</td>
<td>- 65%</td>
</tr>
<tr>
<td>Screening and elimination N (% decrease*)</td>
<td>1,265 (93)</td>
<td>31 (72)</td>
<td>26 (75)</td>
</tr>
<tr>
<td>Compared to base scenario</td>
<td>-88%</td>
<td>- 68%</td>
<td>- 66%</td>
</tr>
<tr>
<td>Focused treatment: ≥F3 N (% decrease*)</td>
<td>12,210 (36)</td>
<td>33 (79)</td>
<td>30 (71)</td>
</tr>
<tr>
<td>Compared to base scenario</td>
<td>+15%</td>
<td>-57%</td>
<td>- 60%</td>
</tr>
<tr>
<td>Focused treatment: ≥F2 N (% decrease*)</td>
<td>9,788 (49)</td>
<td>28 (75)</td>
<td>24 (76)</td>
</tr>
<tr>
<td>Compared to base scenario</td>
<td>- 8%</td>
<td>- 68%</td>
<td>- 68%</td>
</tr>
<tr>
<td>Focused treatment: ≥F0/F1 N (% decrease*)</td>
<td>1,600 (92)</td>
<td>36 (76)</td>
<td>30 (71)</td>
</tr>
<tr>
<td>Compared to base scenario</td>
<td>- 85%</td>
<td>- 63%</td>
<td>- 60%</td>
</tr>
</tbody>
</table>

* Decrease compared to base (from 2014 to 2030). Abbreviations: HCC: hepatocellular carcinoma, LRD: liver-related deaths

Table 4. HCV viremic prevalence according to screening by Birth Cohort

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Tests Required to Identify 1 Viremic Case</td>
<td>833</td>
<td>354</td>
<td>430</td>
<td>322</td>
</tr>
<tr>
<td>Tests Required to Identify 1 New Case</td>
<td>1706</td>
<td>725</td>
<td>880</td>
<td>659</td>
</tr>
</tbody>
</table>
Sensitivity analysis

A sensitivity analysis was conducted to assess the effect of variations in different input and outcome parameters (new infections 2014, treatment 2014, fibrosis stage, progression to HCC and/or liver-related death) on the key driver of uncertainty HCV prevalence (range 0.05 – 0.27 %). Since the model is based on incidence of HCV, the number of new HCV-infections required to match the reported prevalence was calculated in the model. The top driver reflecting the uncertainty in HCV-prevalence is new HCV-infections. This has a direct impact on the forecasted population by disease stage, mortality and disease progression. The impact of all other assumptions was small.

DISCUSSION

Under the current treatment structure, the base scenario, the prevalence of viremic HCV-infection is projected to decrease with 45 % over the next 15 years. This sharp decline is likely attributed to successful treatment of HCV-infection and lower mortality among the ageing population, in combination with low incidence of new HCV-infections. Although transmission of HCV in The Netherlands is low, HCV-related mortality and occurrence of HCC is substantial. Treatment of HCV-infection in an early stage might prevent the occurrence of HCV-related mortality and HCC.

Of all scenarios, the “screening and elimination” scenario predicts the largest reduction of 88 % in viremic HCV-infection prevalence in The Netherlands. This scenario is probably not the most feasible scenario as it requires screening and prevention programs to achieve the inputs required. A more realistic scenario would be the “increased efficacy and treatment uptake” scenario, in which a phased increase of treatment uptake is calculated based upon genotype and fibrosis stage. This scenario predicts a only slightly smaller reduction in viremic HCV infection prevalence compared to the base scenario of 85 %. If we focus on liver-related deaths and HCC, the “≥ F2 only model” provides the greatest decrease from the base scenario (both liver-related deaths and HCC 68 %). However, the decrease in this model is only slightly greater than the decrease predicted by the “screening and elimination model”, the “increased efficacy and treatment uptake model” and the “> F0/F1 model” (68 % for the “≥ F2 only model” versus 60 – 67 % for the other three models). Besides this, the “F2 only model” predicts only a slight reduction in viremic HCV infection prevalence (8 %) whereas the other three models predict a reduction 85 – 88 % compared to the base scenario. All taken together, it seems that the “increased efficacy and treatment uptake” scenario is the most feasible scenario in the current Dutch situation, which also predicts substantial reductions in viremic HCV-infection prevalence, HCC and liver-related deaths. However, due to the current high costs of treatment with DAAs it is very unlikely that this scenario would be adopted in the near future. Lower prices of DAAs are necessary to make this scenario in which a substantial decrease of prevalent HCV-infections can be achieved more realistic.

Most of the described models require an increase in treatment uptake to 1,700 individuals annually, and allowing treatment access to individuals regardless of fibrosis stage. Over the last years there have been about 1,000 (range: 880 – 1,130) treatments with peg-IFN and RBV (with or without DAAs) annually. Assuming that this number is
representative of the number of treatments for HCV, an increase from 1,000 to 1,700 treatments annually may be feasible with the current capacity in The Netherlands as new therapies have a shorter duration and less side-effects. Next to this, it should be noted that the increase in treatment uptake per year is only required for the first eight years. After this initial investment, the yearly treatment drops significantly to 270 patients treated yearly by 2030.

Increases in SVR have the potential to have favourable improvements in end-stage liver disease, with maybe little changes in the ultimate treatment rates. With the new treatment regimens with low side effects, treatment-uptake is likely to increase, and with the high SVR-rates, the need for retreatment will be low. It is known that curing HCV-infection in liver cirrhosis patients reduces complications of cirrhosis and risk of HCC. However, although reduced, these patients are still at risk of decompensation of liver cirrhosis and/or HCC. They are therefore advised to remain in long-term clinical care for monitoring progression of liver disease and/or development of HCC. In the current model these patients have not been considered as continued burden of HCV-infection after SVR. From this point of view it might be worthwhile to treat patients before the stage of cirrhosis, as the risk of HCC following SVR among patients with F0-1-2-3 is negligible. This higher treatment rate (with high SVR rates) for patients with a lower fibrosis stage may have favourable improvements in end-stage liver disease with no changes in the eventual treatment rates and could prevent ongoing transmission. This might save future costs for follow-up of chronic liver disease (cirrhosis) and long-term HCC monitoring. Next to this, achievement of SVR after treatment of chronic HCV reduces non-liver related mortality and (extra-hepatic) manifestations of HCV-infection, and improves quality of life. These are all factors in reducing health care costs related to HCV-infection.

Achievement of our described strategy to treat more HCV-patients is dependent upon the detection of people with HCV-infection, thus, reinforcing the need for increased awareness and intensified screening among risk groups and professionals. One might consider a risk group approach. Alternatively, focusing on a birth cohort of 1960–1964 without prior assessment of HCV risk might be effective as our model suggests that one newly diagnosed viremic case may be found per 659 tests (compared to 1 out of 1,706 for the general population). This approach has been chosen in the USA and was described in 2012. However, the effectiveness and cost-effectiveness of a birth cohort screening strategy or a modified birth cohort screening strategy in which additional risk-based screening criteria are used, need to be determined. Next to this, it is difficult to suggest specific recommendations on birth cohort screening as the age and gender distribution of the viremic HCV-infected population in The Netherlands is not well known.

For first-generation migrants born in countries where HCV-infection is endemic, and other (difficult-to-reach) risk groups for HCV, various pilot screening projects have been performed in recent years, using different screening strategies. However, the (cost-) effectiveness of these strategies relative to each other has not been studied yet, hampering efficient targeting of screening programmes. Moreover, there is no structural screening programme for migrant groups in place and combining HCV screening with screening for other infections might be considered. We suggest that cost-effectiveness analyses of screening strategies targeted at first-generation migrants should
be performed and awareness among risk groups as well as health care professionals should be increased. Increasing knowledge of HCV-infection among health care professionals and the general population may also lower the barriers of testing and referring.\textsuperscript{58}

Next to migrants, two other groups require attention. The first group consists of people that already have been diagnosed with a viremic infection in the past (e.g. HCV-infected blood donors) but have been lost to follow-up in clinical care. The feasibility to retrieve these individuals should be investigated. The second group is more difficult to find because it is hidden in society: individuals that have (occasionally) injected drugs in the past, acquired a tattoo in an endemic region, or received a blood transfusion before 1992. For this group, again, awareness should be increased for both the individuals themselves, and health care professionals, in particular general practitioners and public health workers. Innovative approaches such as internet-mediated blood screening services\textsuperscript{58} might be considered.

There are some factors that limit the value of the described outcomes of the prediction models. First, many parameters that were used as input are based on assumptions or data of less recent years. These data include the current and future number of diagnosed and treated viremic HCV-infections, and the distribution of genotype, fibrosis stage, age and gender of treated and untreated patients. Retrieving actual figures on the different parameters in the Netherlands is very difficult, as there is no national registry of HCV-patients in place. The sensitivity analysis that was conducted with the key driver of uncertainty HCV-prevalence was in turn driven by uncertainty in the number of new HCV-infections. The impact of all other assumptions was small. Second, parameters were not specified per risk group. These groups however have different characteristics, including the proportion diagnosed, genotypes, treatment rates and treatment outcome, influencing the outcome of the models. Third, factors such as sex differences and HIV-infection, and their impact on clearance and HCV disease progression have not been taken into account. Fourth, in this analysis it was assumed that the number of new infections and re-infections remained constant in all scenarios described. While disease progression models can predict disease burden, they are less accurate for estimating future prevalence as they do not explicitly model HCV transmission nor include the possibility of re-infection following successful therapy.\textsuperscript{59} Finally, FibroScan has been used for assessing the fibrosis score as a selection criterion for treatment and defining the different groups for the models. This might not be the most accurate way as FibroScan scores are only reliable in low (F0) and high (F4) ranges, but are not between META VIR scores F1 to F3. Also, FibroScan does not differentiate between META VIR scores F2 and F3. Fibrosis staging in this range should be done using a liver biopsy. These limitations may lead to incorrect inputs and estimations, leading in turn to incorrect predictions. Over time, the inputs of the models may have to be adjusted and updated, and linked with transmission models to achieve correct predictions.

In conclusion, the largest decrease in viremic HCV-infections in The Netherlands may be achieved by applying the “elimination” strategy. Preventing progression of HCV-related liver disease leading to HCV-related death and HCC is best achieved when using the “≥F2 fibrosis” strategy. The most realistic with reasonable reductions in HCV-prevalence, HCV-related death and HCC would be the “increased efficacy and treatment uptake” strategy with a phased increase of treatment uptake. To be able to achieve these future goals, diagnosis of people with HCV-infections in The Netherlands
who may benefit from treatment should be increased. Prevalence data and knowledge regarding facilitating and impeding factors for HCV screening are needed for the largest risk groups separately (including the different migrant groups). Awareness among risk groups and professionals as well as the general population should be increased whereas barriers on different levels (practical, psychological) should be lowered. A coordinated national strategy and sufficient financial means to support it are needed to achieve these goals. The presented models on the future disease burden might inform our national strategy.

ACKNOWLEDGEMENTS

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PART III

HEPATITIS E VIRUS INFECTION
CHAPTER 8

Hepatitis E Virus Infection and Hepatic Graft versus Host Disease in Allogeneic Hematopoietic Stem Cell Transplantation Recipients

* contributed equally

Adapted from:
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ABSTRACT

Hepatitis E virus (HEV) genotype 3 infection has become important for immunocompromised patients, because of their propensity to develop chronic HEV-infection and liver cirrhosis. We retrospectively investigated the incidence of HEV-infection in patients with elevated ALT levels, in a cohort of 130 allogeneic hematopoietic stem cell (alloHSCT) recipients. Of a total of 130 patients, 123 had one or more episodes of elevated ALT-levels. Five out of these 123 patients had HEV-infection (4%). Interestingly, 3 of these patients had signs of concomitant graft versus host disease (GvHD) of the liver. These data demonstrate that HEV-infection is prevalent among alloHSCT recipients and may be related to the presence of GvHD. HEV-infection should be considered in all alloHSCT recipients with elevated ALT-levels, particularly in patients with GvHD of the liver.
INTRODUCTION

Hepatitis E virus (HEV) is a non-enveloped, single stranded RNA virus, which can be subdivided into at least four genotypes. Genotypes 1 and 2 are human viruses causing acute hepatitis, mainly in young adults in tropical countries. Genotypes 3 and 4 are zoonotic, with pigs as the main reservoir in Europe and parts of Asia1. The most prevalent HEV genotype in Europe is genotype 3, which is normally asymptomatic and self-limiting. It does, however, pose a threat to immunocompromised patients who may develop chronic HEV-infection (58-93%)2-5 and liver cirrhosis3,6,7. In a Dutch cohort of 328 allogeneic hematopoietic stem cell transplantation (alloHSCT) recipients, 8 cases of HEV-infection (2.4%) were found of which 5 developed chronic hepatitis8. This suggests that there is a considerable risk of post-transplant HEV-infection for alloHSCT recipients. Most patients with HEV-infection have increased ALT-levels and most alloHSCT patients experience one or more episodes of elevated transaminase levels post-transplantation. The differential diagnosis of elevated liver enzymes including transaminases following alloHSCT is however extensive and includes medication toxicity, pre-existing liver conditions such as fatty liver disease and graft versus host disease (GvHD) of the liver. Moreover, as infections may incite GvHD it can be hypothesized that HEV can provoke GvHD of the liver. Our aim was to identify the prevalence of HEV-infection among alloHSCT patients with elevated ALT-levels.

METHODS

We performed a retrospective analysis of ALT-levels in all 130 patients who received an allogeneic HSCT between January 1st 2005 and April 1st 2015 at our institution. Elevated ALT-levels were defined as: ALT > 50 U/L for at least four consecutive weeks, recurrent elevated ALT-levels > 50 U/L for a shorter period of time with normal ALT-levels in between, or an episode of peaking ALT of > 100 U/L during a period of less than 4 weeks. HEV-RNA was measured at times of ALT-elevation in stored PCR-grade plasma samples using a real-time quantitative PCR amplifying the open reading frame (ORF) 3 region of HEV9. For patients with HEV-infection, additional serial plasma samples were retrieved and tested to follow HEV-infection over time.

RESULTS

Patient characteristics are summarized in Table 1. Of 130 alloHSCT recipients (70 men and 60 women), 123 showed one or more episodes of elevated ALT-levels (total: 147 episodes). Hepatic GvHD was diagnosed or strongly suspected (based on elevated cholestatic liver enzymes in combination with biopsy-proven GvHD of skin or intestine, that responded to steroid-therapy) in 19 patients (16% of the 130 alloHSCT patients). For 141/147 episodes of ALT-elevation a plasma sample was available for HEV-RNA testing. Five samples belonging to 5 different patients were HEV-RNA positive, resulting
### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Allogeneic HSCT patients (n=130)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age – yr</strong></td>
</tr>
<tr>
<td>Mean (range)                 51 (19-72)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td>Male/ Female                70 / 60</td>
</tr>
<tr>
<td><strong>Diagnosis – no. (%)</strong></td>
</tr>
<tr>
<td>AML                         60 (46)</td>
</tr>
<tr>
<td>ALL                         23 (18)</td>
</tr>
<tr>
<td>CML                         5 (4)</td>
</tr>
<tr>
<td>CLL                         7 (5)</td>
</tr>
<tr>
<td>Non Hodgkin Lymphoma        12 (9)</td>
</tr>
<tr>
<td>Hodgkin Lymphoma            3 (2)</td>
</tr>
<tr>
<td>Other                       20 (15)</td>
</tr>
<tr>
<td><strong>Type of allogeneic HSCT – no. (%)</strong></td>
</tr>
<tr>
<td>RIST-sib                    35 (27)</td>
</tr>
<tr>
<td>RIST-MUD                    64 (49)</td>
</tr>
<tr>
<td>MA-sib                      12 (9)</td>
</tr>
<tr>
<td>MA-MUD                      12 (9)</td>
</tr>
<tr>
<td>CB                          7 (5)</td>
</tr>
</tbody>
</table>


### Table 2. Characteristics of HEV-infected patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex, age§ (yr)</th>
<th>Underlying disease</th>
<th>Transplantation type (yr)</th>
<th>Hepatic GvHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M, 60</td>
<td>CLL</td>
<td>MUD-RIST (2006)</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>M, 37</td>
<td>ALL</td>
<td>MA-MUD (2010)</td>
<td>Yes*</td>
</tr>
<tr>
<td>3</td>
<td>F, 70</td>
<td>CLL</td>
<td>MUD-RIST (2011)</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>M, 41</td>
<td>CML</td>
<td>MA-MUD (2012)</td>
<td>Yes*</td>
</tr>
<tr>
<td>5</td>
<td>F, 54</td>
<td>Hodgkin Lymphoma</td>
<td>MUD-RIST (2014)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

§Age at time of HEV diagnosis; *Elevated transaminases and cholestatic liver enzymes have been attributed to hepatic GvHD because they occurred at the time of biopsy-proven chronic GvHD of the gastro-intestinal tract and/or skin. A liver biopsy was not taken.

Abbreviations: CLL: chronic lymphoid leukemia; ALL: acute lymphatic leukemia; CML: chronic myeloid leukemia; MUD: matched-unrelated donor; RIST: reduced intensity stem cell transplantation; MA: myeloablative.
in an HEV-infection prevalence of 4% among alloHSCT recipients with ALT-elevations. All these 5 patients had persistent or recurrent ALT-elevations. Of the five patients with HEV-infection, three had suspicion of concomitant hepatic GvHD; two patients (patient 2 and 4) had biopsy-proven GvHD of the skin and intestine, and suspected GvHD of the liver at the time of HEV infection, in one patient (patient 5) hepatic GvHD was confirmed by a liver biopsy. We describe the case histories of the five HEV-infected patients below (Table 2. and Figure 1).

Patient 1 underwent a reduced intensity alloHSCT (RIST) of a matched unrelated donor (MUD) because of chronic lymphatic leukemia (CLL) in 2006. He developed acute and chronic GvHD of the skin, for which he received prednisolone that could be tapered and finally stopped in 2009. In 2014 a relapse of the CLL occurred which was treated with fludarabine/cyclophosphamide/rituximab (FCR) chemotherapy. This was complicated by chronic varicella zoster infection and herpes simplex infection, for which he received maintenance treatment with valaciclovir and ganciclovir. He was diagnosed with HEV-infection 9 years after alloHSCT, a few months after the last cycle of FCR. Treatment of HEV-infection with ribavirin was initially successful, but after stopping therapy a relapse occurred. Also a second course of therapy did not result in clearance of the virus.

Patient 2 received a myeloablative (MA) MUD alloHSCT because of acute lymphatic leukemia (ALL) in 2010. Three months after alloHST he developed cytomegalovirus (CMV) reactivation, which was treated with valganciclovir, and acute, biopsy-proven GvHD of the skin and intestine which was treated with prednisolone. During this time, ALT-levels were elevated, but they normalized upon treatment for CMV-reactivation and GvHD. Three years later, during an exacerbation of chronic GvHD of the skin and intestine

Figure 1. Graphic representation of the three patients with HEV-infection and suspected or diagnosed hepatic GvHD (patient 2, 4 and 5). See for detailed description of patient histories the main text.
(biopsy-proven), he was diagnosed with HEV-infection (Figure 1a). GvHD of the liver was considered because of elevated transaminases, however, this was not confirmed by a liver biopsy. HEV-infection was treated successfully with ribavirin and the transaminases normalized subsequently.

Patient 3 received a MUD-RIST because of CLL in 2011. The transplantation was complicated by chronic GvHD of the skin and oropharynx, which was treated with prednisolone, cyclosporin, imatinib and eventually, in 2014, rituximab. She also had disseminated varicella zoster and herpes simplex virus infections for which she received valaciclovir. HEV-infection occurred after rituximab therapy, and was successfully treated with ribavirin. The patient eventually died from recurrent opportunistic respiratory tract infections with 

Patient 4 received a MA-MUD HSCT because of blast crisis chronic myeloid leukemia (CML-BC) in 2012. The transplantation was complicated by acute and chronic steroid-refractory biopsy-proven GvHD of the intestine, which was treated with mesenchymal stem cell transplantation resulting in partial remission. He had peaking ALT-elevations during this period which was attributed to GvHD, iron deposition and medication toxicity. A liver biopsy was not performed. Retrospective analysis of stored plasma samples revealed that he acquired HEV-infection before alloHSCT. HEV-infection and GvHD remained active until he succumbed to systemic yeast infection 16 months after alloHSCT (Figure 1b). In this patient, HEV-infection was diagnosed after his death, and therefore it was left untreated.

Patient 5 received a MUD-RIST because of relapsing Hodgkin’s lymphoma in 2011, which was complicated by GvHD of the skin and liver. At the time of diagnosis of GvHD, the patient was also diagnosed with HEV-infection (Figure 1c). A liver biopsy taken at that time showed an irregular morphology of the bile duct epithelium, consistent with GvHD. Moreover periportal infiltrates were seen together with some lobular inflammation and
Councilman bodies (apoptotic bodies), the latter fitting with both hepatic GvHD and HEV-infection (Figure 2). Analysis of stored plasma samples revealed that HEV-infection preceded GvHD of the liver, suggesting that HEV-infection may have provoked this allo-immune response. The patient responded well to treatment with ribavirin but eventually succumbed to relapsed Hodgkin’s lymphoma.

DISCUSSION

The prevalence of HEV-infection was 4 % in this cohort which is higher than was reported for the general population (0.13 %)\(^{10}\), for solid organ transplant patients (1 – 3 %)\(^{6}\), for HIV-patients (0.12 %)\(^{11}\), and for another Dutch alloHSCT cohort (2.4 %)\(^{8}\). The latter study was performed between 2006 and 2011, while our study was conducted between 2005 and 2015. All HEV-infections in our cohort occurred after 2012, which fits with the observation that HEV-prevalence in Europe is rising\(^{12}\) and offers an explanation for the higher incidence we observed. As is recommended in recently published guidelines for treatment of viral hepatitis in patients with hematological disorders\(^{13}\), infection with HEV should be considered in these immunocompromised patients, especially those with chronic lymphopenia, who are at-risk for chronic HEV-infection with rapid advancement to liver cirrhosis.

We observed that HEV-infection coincided with (suspected) hepatic GvHD in three of five HEV-RNA positive patients. In this study population of 130 patients, 19 patients had (suspected) hepatic GvHD, of which 3 (16 %) had concomitant HEV-infection. Of the 111 patients without hepatic GvHD only 2 (2 %) had HEV-infection. GvHD is characterized by an immune response of donor lymphocytes against host tissues. Intense conditioning procedures or local damage, for example caused by infection, may induce such allo-immune responses and GvHD\(^{14}\). Therefore, one can hypothesize that HEV-infection can also initiate or maintain (hepatic) GvHD. In two out of the three patients (patients 2 and 5) with concomitant HEV-infection and suspicion or diagnosis of hepatic GvHD, treatment with ribavirin led to rapid clearance of the virus in parallel with resolution of GvHD. In one patient (patient 4) with concomitant HEV-infection and (suspected) hepatic GvHD, HEV-infection was not diagnosed and therefore left untreated. GvHD in this patient was therapy-refractory.

AlloHSCT recipients often demonstrate elevated ALT-levels, a sign of liver tissue damage that is usually ascribed to drug toxicity, iron deposition, infection, sinusoidal obstruction syndrome (SOS) or GvHD\(^{15}\). Hepatic GvHD is difficult to distinguish from other liver diseases, and our observation that 16 % of patients with (probably or proven) hepatic GvHD had concomitant HEV infection underlines the importance of serologic testing of these patients.

In conclusion, allogeneic HSCT recipients are at high risk of liver disease, including HEV-infection. It may cause chronic liver inflammation ultimately leading to cirrhosis, and our observations led us to hypothesize that HEV can provoke or sustain hepatic GvHD. While our results need further study and confirmation in larger alloHSCT cohorts, data from our and another group\(^{8}\) demonstrate that HEV-infection should be considered in all alloHSCT recipients with persistently elevated ALT-levels, particularly in those with concomitant hepatic GvHD.
REFERENCES

Summary and General Discussion
SUMMARY

The described work comprises different clinical aspects of viral hepatitis B, C and E infection. First, the immune response during acute hepatitis B is investigated. Next, intrahepatic and plasma IP-10 levels are analysed as a marker of response to peginterferon-based therapy in chronic hepatitis B and C virus infection. Furthermore, various treatment options for chronic hepatitis B, C and E are discussed. Moreover, the hepatitis C-related disease burden in 2030 is predicted by using a modelling approach. Finally, the prevalence of chronic hepatitis E virus infection in allogeneic hematopoietic stem cell transplantation patients and its clinical relevance is studied.

Part I – Hepatitis B Virus Infection

Chapter 1 describes the immune responses of 9 patients with acute HBV-infection, in terms of NK-cell characteristics and HBV-specific T-cell function. Of these 9 patients, one became chronically infected and the other 8 cleared the virus within 6 months. Early time points after infection showed an increase in CD56bright NK-cells, and in the proportion of cells expressing markers of activation. Most of these normalised at week 24, while the proportion of TRAIL-positive CD56bright NK-cells remained high in the chronically infected patient. In patients that cleared HBV, functional HBV-specific CD8+ and CD4+ T-cell responses were observed, whereas in the patient who developed chronic infection, only low HBV-specific T-cell responses were found at all time points. This indicates that NK-cells are activated early during acute HBV-infection. In patients who clear acute HBV-infection, broad and multi-specific T-cell responses are observed. As exemplified by one patient who did not clear HBV-infection, failure of NK-cell normalisation as well as narrow T-cell responses may be indicative of chronic infection.

In Chapter 2, the role of pre- and on-treatment IP-10 levels in chronic hepatitis B (CHB) patients with high viral load, treated with a combination of peginterferon (pegIFN) and a nucleo(s)tid analogue (NA) is investigated. It was found that plasma IP-10 levels and IP-10 mRNA expression in the liver at baseline were correlated with one another, especially in HBeAg-positive patients. Higher pre-treatment IP-10 levels in plasma were associated with combined response (HBeAg-loss, ALT-normalization and HBV-DNA < 2,000 IU/mL) in HBeAg-positive, but not in HBeAg-negative CHB patients. Furthermore, there was a correlation between plasma and intrahepatic IP-10-levels and various markers of response such as ALT, HBV-DNA levels and HAI-score of the liver.

Chapter 3 describes a randomized controlled trial in CHB patients with a low viral load who received either pegIFN and adefovir, PegIFN and tenofovir for 48 weeks, or no treatment. The primary objective was HBsAg-loss. In this study, there was no significantly higher rate of HBsAg-loss at Week 72 in patients who were treated with combination therapy, compared to those who received no treatment. However, there was a strong decline in HBsAg of > 1log10 IU/mL observed in 21% of patients treated with combination therapy with pegIFN and adefovir or tenofovir, versus no change in patients who did not receive treatment.

Part II – Hepatitis C Virus Infection

Chapter 4 describes the dynamic changes of plasma IP-10 levels in chronic hepatitis C (CHC) patients treated several years ago, with a high-induction dose of standard
interferon (IFN) for 6 weeks, followed by treatment with pegIFN, ribavirin (RBV) and amantadine. There was no significant relation between pre- or on-treatment plasma IP-10 levels and sustained virological response (SVR). However, IP-10 levels at Day 1 of treatment increased significantly and this increase was related with a decline in HCV viral load at Day 1 of >2.28log10. The increase of IP-10 levels from baseline to Day 1 was significantly greater in patients with a favourable IL28B genotype compared to patients with non-favourable IL28B genotypes. Furthermore, the rise in IP-10 from baseline to Day 1 was dependent on baseline IP-10 levels. In patients with a low baseline IP-10 of <150 pg/mL, an almost 30-fold rise was seen at Day 1, whereas in patients with a high baseline IP-10 of >600 pg/mL only a four-fold rise was observed.

Chapter 5 describes the results of a retrospective multicentre observational real-life cohort study of 53 patients with CHC genotype 4 and advanced liver fibrosis or compensated cirrhosis. The patients were treated with sofosbuvir and simeprevir with or without RBV. An SVR-rate of 92% was observed, which was in line with the data shown in large registration studies in CHC patients with HCV genotype 1. Whether or not the addition of RBV in cirrhotic patients leads to a higher SVR-rate in genotype 4 patients cannot be concluded from our study. In our cohort, all 12 patients who were treated with simeprevir, sofosbuvir and RBV achieved SVR whereas the four patients with a viral relapse did not receive RBV.

Chapter 6 describes a case of a patient with CHC genotype 1a and advanced liver fibrosis, who failed previous therapy with pegIFN and RBV, and subsequently relapsed after therapy with a combination of a NS5A and NS3 inhibitor during 24 weeks in a clinical trial. NS5A as well as NS3 resistance-associated substitutions (RAS) were present and explained the failure of the treatment. After 18 months, NS5A RAS remained abundantly present, but NS3 RAS had disappeared. Whilst waiting for registration and reimbursement of DAAs, the patient underwent gastric bypass surgery for morbid obesity. This patient achieved SVR after treatment with sofosbuvir, simeprevir and RBV for 24 weeks. The dose of simeprevir was adjusted based on therapeutic drug monitoring (TDM), as simeprevir levels were lower than described in literature due to the gastric bypass surgery.

Chapter 7 predicts the effect of different treatment strategies (using a modelling approach) on the HCV-viremic population and related disease burden (defined as liver-related mortality and incidence of hepatocellular carcinoma (HCC)) in 2030. The treatment strategies used comprise various scenarios: starting from a “base scenario”, the amount of CHC patients treated with antiviral therapy is gradually increased based on different criteria. The most important scenario’s that were analysed to predict the HCV-viremic population and disease burden in 2030 were:

A. “base-scenario”: CHC patients with severe liver fibrosis or cirrhosis (META VIR ≥F3/F4) are treated with IFN-based therapy;
B. “increased efficacy”: the same criteria as the base scenario, but treatment with DAAs instead of IFN-based therapy;
C. “all patients”: all patients are treated with DAAs, regardless of fibrosis-stadium;
D. “screening and elimination”: all patients are treated with DAAs, with an increased amount of patients with new HCV-diagnoses as input, and a lower amount of acute HCV-infections due to preventive measures.
The HCV-viraemia prevalence in The Netherlands used to calculate the base scenario is 0.12 %, which is the most recently established prevalence at the time of conducting the study (2013/2014). According to the base scenario, the HCV-viremic population would decrease by 45% by 2030. This would decrease by 85% if the number of treated patients would be increased to all patients (scenario C). Furthermore, the number of individuals with HCC and liver-related deaths are estimated to decrease by 19% and 27% respectively in the base scenario; both patient groups are expected to further decreased by 60–68 % when extending treatment to all CHC-patients (C). With all analysed strategies, a significant reduction in the burden of HCV-viremic infections, liver-related deaths and HCC was predicted.

Part III –Hepatitis E Virus Infection

Chapter 8 describes the prevalence of chronic HEV-infection in patients who underwent allogeneic hematopoietic stem cell transplantation (alloHSCT) in The Netherlands. Chronic HEV-infection was established in 5 out of 130 patients (4 %), which is higher than was reported in other cohorts of immunocompromised patients, such as solid organ transplant recipients and HIV-patients (0.12–3 %) in The Netherlands. There was a possible relation between chronic HEV-infection and hepatic graft versus host disease (GvHD), as in three of the five patients with chronic HEV-infection hepatic GvHD was suspected. In two of these patients, treatment with RBV led to rapid clearance of the virus and resolution of GvHD. In the third patient, the HEV-infection was (retrospectively) diagnosed after the patient had died and therefore left untreated. GvHD in this patient was therapy-refractory. In conclusion, a higher prevalence of chronic HEV-infection in alloHSCT patients than was reported earlier in immunocompromised patients in The Netherlands was observed, and these findings may suggest a relation between hepatic GvHD and chronic HEV-infection.
GENERAL DISCUSSION

Immunological phenomena in HBV-infection

The mechanism of developing chronic HBV-infection, rather than clearing the infection, is not fully understood but is likely multifactorial. The ability to control (and clear) an acute HBV-infection is thought to be related to the balance between the quantity of infected hepatocytes and the efficiency of the T-cell response. This T-cell response may be attenuated by several factors that could be divided into self-save\textsuperscript{1-3} and viral escape mechanisms.\textsuperscript{4-10} Development of chronic hepatitis B (CHB) is associated with impairment of the innate and adaptive immune responses and with narrow and weak T-cell responses.\textsuperscript{1-3,6,8,9,11} It remains a question of contention whether the impairment of T-cell response is a cause or consequence of persistent infection.

It was earlier shown that HBV-specific T-cell responses in CHB patients with low viral load are relatively narrow and only directed towards the HBV core antigen (HBcAg). These T-cells have a strong proliferative ability by which they supposedly keep HBV replication at a low level. In CHB patients with high viral load, HBV-specific T-cell responses are low. A partial recovery of the HBV-specific T-cell response was observed in patients with HBsAg-loss or long-term viral suppression (with NA's).\textsuperscript{11} NK-cells play a pivotal role in the clearance of HBsAg during interferon (IFN) - based therapy. Their phenotype and function are significantly altered during and after treatment with interferon.\textsuperscript{12} The findings (Chapter 1) that NK-cells are highly activated during acute HBV-infection, and that HBV-specific CD8\textsuperscript{+} and CD4\textsuperscript{+} responses in patients who clear the virus, are broad and functional, confirms the suggestion that these immune reactions are very significant for clearance of the virus. Whether or not these findings are cause or consequence of the HBV-infection remains an open question and a topic for further research.

In Chapter 2 it is described, that plasma IP-10 levels and IP-10 mRNA expression in the liver are correlated with each other in HBeAg-positive CHB patients. Higher pre-treatment IP-10 levels in plasma are associated with combined response (HBeAg-loss, low HBV-DNA (< 2,000 IU/mL) and normal ALT) in HBeAg-positive, though not in HBeAg-negative CHB patients. This suggests that IP-10 reflects intrahepatic immune activation. The fact that IP-10 mRNA expression in the liver was also associated with plasma ALT-levels supports this hypothesis. Given the function of IP-10 being a chemo-attractant for inflammatory cells, high intrahepatic IP-10 expression is essential for migration of leukocytes into the liver in response to a viral infection. In HBeAg-positive patients, pre-treatment immune activation is important for further induction of the innate immune system, thus inducing a virological response. IP-10 kinetics shortly after start of peginterferon (pegIFN)-based treatment, showed a significant two- to threefold rise in IP-10 plasma levels at Day 3 after start of treatment, irrespective of HBeAg-status or response to therapy. This indicates that early after start of pegIFN-based treatment, the immune activation of responders and non-responders was similar. In conclusion, our findings suggest that the increase in IP-10 levels early after start of pegIFN-based treatment is the result of a non-specific stimulation of the IFN type I immune response. This causes the induction of multiple interferon-stimulated genes (ISGs) which provoke in turn response to therapy.
HBV treatment and future perspectives

Presently, the closest to a cure for CHB is loss of HBV-DNA and HBsAg with or without formation of anti-HBs antibodies, also named functional cure. However with a functional cure HBV is still integrated in the nucleus of the hepatocyte as cccDNA. Therefore in the future also removal of cccDNA in the cell, also named total or chemical cure, is the ultimate goal for treatment of CHB patients.

Combined response to current therapy is defined as HBeAg-loss, low HBV-DNA (< 2,000 IU/mL) and normal ALT in HBeAg-positive CHB patients; or low HBV-DNA (< 2,000 IU/mL) and normal ALT in HBeAg-negative CHB patients. Current guidelines for treatment of CHB recommend treatment with either a finite course of pegIFN or long-standing nucleos(t)ide analogue (NA) therapy. The aim of these treatment modalities is maintained viral suppression. The decision to treat a patient who has a high HBV-DNA load and active liver inflammation is obvious, as the risk to develop liver cirrhosis and possibly hepatocellular carcinoma (HCC) if untreated is substantial. For HBeAg-negative CHB patients with low viral load (<2,000 IU/mL) and low inflammatory activity (normal ALT), treatment is not indicated, although these patients are at increased risk of liver cirrhosis and development of CHB. However, there are no treatment intervention studies available on this topic in CHB patients with low viral load. An earlier study in HBeAg-negative CHB patients with high viral load (> 2,000 IU/mL) treated with a combination of pegIFN and lamivudine showed disappointing results regarding HBsAg-loss.

In a recent randomised controlled study 740 HBeAg-positive and -negative CHB patients with high viral load were randomly assigned to treatment with a combination of pegIFN and tenofovir, or pegIFN, or tenofovir alone. This study showed promising results for the combination therapy with HBsAg-loss of 9.1 % compared to 2.8 % with either monotherapy (tenofovir or pegIFN alone). A previously conducted study showed a relatively high rate of HBsAg-loss among CHB patients with high viral load (HBeAg-positive and HBeAg-negative) treated with a combination of pegIFN and adefovir. The results of those studies suggest that combination therapy in patients with high viral load is superior to pegIFN monotherapy, with respect to HBsAg-loss. Response to combination therapy was associated with low baseline HBsAg-levels, a feature that is not useful for patients with low viral load, as they characteristically already have a low HBsAg-level. Apparently, the antiviral immune response in those patients keeps HBV-DNA and HBsAg at low levels as T-cell exhaustion is less severe when HBV-DNA levels are low. This suggests, that up-regulating the innate and adaptive immune response by antiviral treatment in CHB patients with low viral load could be successful in terms of viral clearance. As there were no studies available assessing treatment in HBeAg-negative CHB patients with low viral load, we decided to set-up a randomised controlled study with the aim to assess HBsAg-loss during or following therapy with combination therapy (pegIFN plus adefovir or tenofovir versus no treatment), and to establish markers of response to therapy. The results were that we did not find a higher rate of HBsAg-loss in the group of HBeAg-negative CHB patients with low viral load who were treated with combination of pegIFN and a NA (adefovir or tenofovir) compared to patients who received no treatment (Chapter 3). However, we did observe a strong decline in HBsAg of >1log10 IU/mL in 21 % of patients treated with combination therapy with pegIFN and adefovir or tenofovir, versus no change in HBsAg-levels in patients who did not
receive treatment. Possibly a longer duration of follow-up of these patients may show whether the strong decline in HBsAg-levels in the treatment arms eventually may lead to higher rates of HBsAg-loss. In patients who had a strong HBsAg decline, a marked rise in ALT-level after start of therapy was observed, and a rebound in HBsAg-level after stopping treatment. This indicates a possible enhancement of the antiviral immune response due to the treatment. These patients are an interesting group to study further, enabling the selection of CHB patients with a low viral load who could benefit from pegIFN-based therapy.

Different treatment strategies might also be considered, such as add-on therapy of pegIFN to NAs,\textsuperscript{21} switch from NA therapy to pegIFN,\textsuperscript{22} or therapeutic vaccination added to either pegIFN or NAs.\textsuperscript{23} A recent study in HBe-negative CHB patients, who were treated with NAs for at least one year and who had undetectable HBV-DNA, showed that addition of a 48 week course of pegIFN was tolerated poorly and did not result in a significant increase of HBsAg clearance.\textsuperscript{24} An additional analysis of this study showed that HBsAg decline at week 24 might be useful to identify patients who may benefit from add-on treatment with pegIFN eventually leading to HBsAg-loss.\textsuperscript{25}

In the study described in Chapter 3, CHB patients who have no or little inflammatory activity, and a limited increased health risk, are treated with a very toxic treatment with only a small chance of success. To apply this to clinical practice, is in the writer’s opinion, a bridge too far. However, it is of great importance to find a finite curative treatment for CHB, also for the group of CHB patients with low virus activity, to minimize health care costs, risk of reactivation, and overcoming possible stigmata.

New compounds, currently under development, are directed against the virus itself (cccDNA targeted therapy, inhibition of capsid assembly, polymerase inhibitors, RNA interference and HBsAg production targeted therapy), the host proteins involved (entry inhibitors, microRNA’s, FXR-agonists) or the host immune response (TLR agonists).\textsuperscript{26–29} Combining different treatment modalities that act against the replication of the virus and enhance immune response would hopefully result in higher rates of functional cure than with current therapy. The ultimate goal is to achieve a total cure, including the elimination of cccDNA from the nucleus of the hepatocyte. However, this therapy is not expected in the next decade.

**Response markers in chronic HCV-infection**

Multiple inflammatory chemokines and cytokines have been suggested as markers for treatment outcome because of their regulatory function in the HCV-specific immune response. Most of these cytokines are modulated by exogenous IFN and play a critical role in viral clearance.\textsuperscript{30} After an infection with HCV, the innate immune system initiates a non-specific immune response through type I IFN, leading to the activation of the intracellular pathway resulting in the induction of multiple ISGs, among which the gene encoding IP-10.\textsuperscript{31,32} This cytokine, and especially its relation to IL28B polymorphisms, viral clearance and response to antiviral therapy, has been described in CHC.\textsuperscript{33}

In Chapter 4 a high rise in plasma IP-10 levels shortly after administration of high-dose interferon\textsuperscript{34} is described in CHC patients. This might in part be caused by a dose-dependent effect of IFN. However, the fact that the amount of this rise is dependent of baseline IP-10 levels, suggests an important role of pre-treatment activation of the innate immune system in CHC, which is in turn closely related to IL28B genotype.
To conclude, in CHC, low pre-treatment activity of the innate immune system appears to predispose to a higher educability of activity due to IFN-based treatment. These observations on IP-10 in CHC have eventually led to a better understanding of the mechanisms of clearance of the viral infection. However, for predicting the treatment outcome for CHC, measurement of plasma IP-10 level has no relevance today.

**HCV treatment and future perspectives**

Since the introduction and registration of DAAs for the treatment of CHC in 2014, the perspective of HCV treatment has totally changed. DAAs are highly effective in eradicating HCV-infection, and have little side effects. Shortly after registration in 2014, DAAs were not widely reimbursed because of their high costs, but were only available for patients with advanced liver fibrosis (Metavir F3 score) or liver cirrhosis (Metavir F4 score). Based on those reimbursement criteria and the fact that limited literature was available on the treatment with DAA's of genotype 4 CHC patients, patients with genotype 4 CHC and advanced fibrosis or cirrhosis were selected for treatment with sofosbuvir and simeprevir, with or without ribavirin (RBV) for 12 weeks. In the EASL HCV treatment guidelines these patients are not excluded from the recommendation for treatment with sofosbuvir or simeprevir despite the lack of studies showing the efficacy of such treatment.35 Chapter 5 describes the results of a retrospective multicentre observational study in a real-life cohort of 53 patients showing sustained virological response (SVR) in 92% of these patients. This was the first study to show efficacy of the combination treatment with sofosbuvir and simeprevir in patients with CHC genotype 4 and advanced liver fibrosis or cirrhosis. Whether or not the addition of RBV in cirrhotic CHC patients leads to a higher SVR-rate in genotype 4 patients remains unknown. In the described study cohort, all 12 patients who were treated with simeprevir, sofosbuvir and RBV achieved SVR, whereas the four patients with a viral relapse did not receive RBV. Although the numbers were relatively small to see statistically significant differences, the addition of RBV to the 12-week regime of sofosbuvir and simeprevir could be considered in treatment-experienced genotype 4 CHC patients with advanced fibrosis or cirrhosis. At present, there are more all-oral treatment options with DAAs available for genotype 4 CHC, mainly based on the combination of sofosbuvir and NS5A inhibitors. Although there seems to be no relevant difference in terms of efficacy between the different available treatment options for genotype 4 CHC, it is worthwhile having the combination of simeprevir and sofosbuvir (+/-RBV) ready as an efficacious and registered treatment option, especially in case of NS5A resistance, as all other DAA combinations contain NS5A inhibitors.

Some scenarios described in Chapter 7 (e.g. offering treatment to all HCV-infected individuals) are close to the current situation in The Netherlands, as all CHC patients are now treated without restrictions. This means, that should the predictions made in Chapter 7 be correct, 85% of all CHC patients in The Netherlands would soon be cured. The remaining 15% would then consist of those who failed DAA therapy (due to viral resistance or non-compliance), those who are unable to be treated with DAAs (unwillingness to be treated, or due to comorbidity), and those who have not yet been diagnosed with CHC. To eradicate CHC in The Netherlands, the undiagnosed HCV viremic population should be found, which is easiest achieved by screening the whole population. Based on cost-effectiveness-analyses, the Dutch Health Council advised
in 2016 to not screen the whole population but only certain risk groups such as first generation migrants from HCV endemic countries, PWID and MSM. It was shown earlier in Western and Asian countries that treatment of CHC lowers all-cause mortality and hepatocellular carcinoma (HCC) incidence and is cost-effective for all CHC patients. Therefore, population-based screening should be considered, since treatment has dramatically improved.

With the further development of even newer pan-genotypic regimens with higher genetic barriers to develop resistance, one may hypothesize that in CHC, HCV genotyping and quantification of HCV load may not be necessary anymore. The case history described in Chapter 6 illustrates the usefulness of resistance-associated substitutions (RAS) measurement in patients with previously failed DAA therapy. Chapter 6 also underlines that although the relatively new DAAs have been well-studied, information about pharmacokinetics or treatment success in different special (rare) populations are still not well known. Therapeutic drug monitoring (TDM) may be helpful in those special cases.

There are still several issues that require future studies, such as optimal timing of treatment of acute HCV-infection, and pre- or post-exposure prophylaxis in high-risk groups such as MSM. Another issue is the question whether or not patients with an SVR should be further medically controlled and, if so, for how long. Liver stiffness measured using Fibroscan seems to decrease after successful treatment of CHC, with both IFN-based and DAA therapy. This also seems the case for some patients who pre-treatment have liver stiffness measurements in the cirrhotic range, dropping post-treatment to a value below the threshold of cirrhosis. What effect this drop in liver stiffness has on HCC-risk is unknown and until that time, those patients should still be controlled in the clinic as cirrhotics.

A recent point of discussion is the occurrence and prognosis of HCC in CHC patients after treatment with DAAs. Treatment with IFN-based therapy ameliorates prognosis of HCC. Various studies in CHC patients treated with DAAs showed either a higher recurrence rate of earlier curatively treated HCC or no difference in incidence of HCC. One may hypothesize that by taking away inflammation by successful treatment of CHC, (whether this is with IFN-based therapy or with DAAs) prognosis of HCC ameliorates. However, this hypothesis has not been studied for treatment with DAAs. Future research is needed in large patient groups with long-term follow-up after successful treatment of CHC with DAAs to clarify these issues.

**HEV-Infection**

It is common knowledge that HEV-infection with genotype 3 is asymptomatic and self-limiting in immunocompetent individuals. However, this poses a threat to immunocompromised patients who are at risk to develop chronic HEV-infection and liver cirrhosis. A higher prevalence of chronic HEV-infection was reported of 4% in allogeneic hematopoietic stem cell transplantation (alloHSCT) recipients than was earlier described. There is a possible relation between chronic HEV-infection and hepatic graft versus host disease (GvHD) as in three out of the five patients with chronic HEV-infection hepatic GvHD was suspected. However, hepatic GvHD is difficult to distinguish from other liver diseases, particularly in the
absence of a liver biopsy. This possibly has led to an overestimation of hepatic GvHD prevalence in our cohort. Nevertheless, this leads to the hypothesis that HEV-infection may in two ways affect alloHSCT recipients: by causing chronic liver inflammation ultimately leading to cirrhosis, and by provoking or sustaining hepatic GvHD. With the increasing prevalence of HEV-infection in Europe, this infection should be considered in all alloHSCT recipients and other immunocompromised patients with persistently elevated ALT-levels, particularly in those with concomitant hepatic GvHD.

Treatment with RBV was an effective therapy for most of the patients we describe in Chapter 8 with chronic HEV-infection. Possible treatment options described in literature are lowering the immunosuppressant therapy, which is often dangerous and therefore ill advised, or treatment with pegIFN or RBV. Due to the toxicity profile, RBV is the most frequently chosen treatment option. RBV is often dosed as 600 mg per day, based on a case series described in 2014. This dosage seems to be too low, as from the writer’s experience, frequent relapses after stopping treatment with this dose are observed. The duration of therapy is another point of discussion. Most cases describe a 3-6 month duration of treatment. A recent study showed an SVR of 63% after treatment for 3 months. This study also showed that a decreased HEV-RNA of 0.5 log copies/mL 1 week after start of treatment had an 88% positive predictive value in predicting SVR. Another recent study showed that protracted fecal shedding of HEV-RNA may predict treatment failure. Patients with chronic HEV-infection treated for 3 months with RBV who had still HEV-RNA detectable in their stools (but not in plasma) at end of treatment all had a relapse after stopping therapy. Treatment of chronic HEV-infection with RBV should be high-dose (1000–1200 mg daily if tolerated), and response-guided, based on HEV-PCR in stool, with continuation of treatment at least 2 months after HEV-RNA is first negative in stools. For patients who are still refractory to treatment with RBV, new treatment options with sofosbuvir, nucleoside analogues, or possibly monoclonal antibodies against the ORF3 protein of HEV are of interest for further research.

In conclusion, as the prevalence of HEV-infection in Europe is increasing, prevalence of chronic HEV-infection among immunocompromised patients is expected to be significant as well. As untreated chronic HEV-infection may rapidly result in liver cirrhosis, awareness among treating physicians of immunocompromised patients is essential, especially when ALT-levels are elevated. Future research will be focused on treatment of chronic HEV-infection, such as dosage, duration, and response guidance of therapy with RBV, direct-acting antiviral agents, or nucleoside analogues. Immunocompromised patients should be advised to avoid consumption of undercooked meat products, especially porcine.
REFERENCES


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Hepatitis B Virus Infectie

HBV is een dubbelstrengs DNA virus wat behoort tot de Hepadnaviridae. Er zijn wereldwijd bijna 350 miljoen mensen chronisch geïnfecteerd met het virus, waarvan ongeveer 50.000 in Nederland. Transmissie van HBV geschiedt op verschillende manieren, waaronder seksueel, verticaal (perinataal) en via bloed-bloed contact. Perinatale transmissie leidt in veel gevallen tot een chronische HBV-infectie. Een infectie met HBV op latere leeftijd wordt in de meeste gevallen geklaard (90 – 95%). Na klaring blijft het virus in de levercel aanwezig. Daarom wordt dit geen genezing, maar “functionele genezing” genoemd, waarbij er geen ontsteking van de lever meer plaatsvindt, virale factoren in bloed zoals HBV-DNA en HBsAg ondetecteerbaar laag zijn, en er meestal specifieke anti-HBs antistoffen gevormd zijn. Bij patiënten met een functionele genezing die immuunsuppressiva gebruiken, kan het immuunsysteem dusdanig geremd worden, dat het virus weer “reactiveert”.

Bij een chronische HBV-infectie is de afweer van de gastheer niet in staat het virus te klaren. Er zijn meerdere mechanismen verondersteld waardoor dit zo is. Om schade te beperken creëert het immuunsysteem een “immuun-tolerante” status waarbij HBV-specifieke T-cellen worden verwijderd door NK-cellen en cytotoxicische T-cellen worden omgezet in een regulator type T-cel. Dit verzwakt de immuunreactie die nodig is om het virus te klaren. Daarnaast is het HBV in staat het immuunsysteem te ontwijken. Een derde mechanisme wordt ook wel T-cel uitputting genoemd, waarbij de gedachte is dat dit veroorzaakt wordt door herhaalde T-cel stimulatie en een gebrek aan activatiesignalen door T-helper cellen.

Behandeling van chronische HBV-infectie heeft tot doel progressie van leverschade, met als gevolg levercirrose en hepatocellulair carcinoom (HCC), te voorkomen. Momenteel zijn er twee soorten medicijnen beschikbaar voor de behandeling van een chronische HBV-infectie: peginterferon (pegIFN) en nucleo(s)tide analogen (NA’s). Ideaaliter leidt behandeling tot functionele genezing, iets wat maar in een klein aantal gevallen lukt. Daarom wordt er niet alleen gezocht naar verschillende voorspellers van respons op de huidige behandelopties, maar vooral naar nieuwe antivirale middelen met een ander werkingsmechanisme dan de bestaande middelen.

Hepatitis C Virus Infectie

HCV is een enkelstrengs RNA virus behorend tot de Flaviviridae. Wereldwijd zijn er meer dan 170 miljoen mensen geïnfecteerd met een chronische HCV-infectie. Geschat wordt dat in Nederland ongeveer 28.000 mensen geïnfecteerd, of geïnfecteerd geweest zijn met HCV. Transmissie van HCV geschiedt voornamelijk via bloed-bloed contact.

Na infectie met HCV reageert het immuunsysteem met een non-specifieke respons via type I interferon wat leidt tot inductie van diverse interferon-gestimuleerde genen (onder andere coderend voor het chemokine IP-10) en activatie van verschillende afweercellen zoals NK-cellen, macrofagen, dendritische cellen en T-lymfocyten. Het gevolg is een sterke specifieke CD4+/CD8+ T-cel respons, met idealiter klaring van het virus. Echter, dit gebeurt maar in 20 – 25% van de gevallen; in 75 – 80% van de gevallen.


Hepatitis E Virus Infectie
HEV is een enkelstrengs RNA virus wat onderverdeeld kan worden in 4 genotypes. Genotype 1 en 2 veroorzaken een acute hepatitis, veelal bij jonge volwassenen in tropische landen. Genotype 3 en 4 zijn zoönose, waarbij varkens het grootste reservoir zijn in Europa en Azië. Transmissie van HEV is via de faecaal-orale route, en verondersteld wordt dat men met HEV genotype 3 en 4 geïnfecteerd kan raken door consumptie van onvoldoende verhitte met HEV besmette vleesproducten. Het meest voorkomende HEV genotype in Europa is genotype 3.

Na infectie met HEV onstaat een sterke humorale immuunrespons, waarbij IgM antistoffen detecteerbaar worden, enkele dagen later gevolgd door IgG antistof response. IgM antistoffen verdwijnen na enkele maanden, IgG kan jaren aantoonbaar blijven. Bij immuungecompromitteerde patiënten is de HEV antistof bepaling onvoldoende om een viremie uit te sluiten en dient HEV-RNA bepaald te worden.

Een infectie met HEV genotype 3 verloopt meestal asymptomatisch, en wordt doorgaans vanzelf geklaard. Immuungecompromitteerde patiënten kunnen daarentegen een chronische HEV-infectie ontwikkelen, wat snel tot levercirrose kan leiden. Bij patiënten met een allogene hematopoietische stemceltransplantatie is het vóórkomen van een chronische HEV-infectie beschreven. Daarnaast treden er regelmatig leverenzymafwijkingen op als gevolg van medicatie, pre-existentie leverziekten zoals non-alcoholische vetleverziekte, graft versus host disease (GvHD) of andere virusinfecties.

Onderkenning van een chronische HEV-infectie is belangrijk, zodat behandeling overwogen kan worden. De eerste behandelingsoptie van chronische HEV-infectie is het verlagen van immunosuppressiva. Indien dit niet mogelijk is, wat vaak het geval is, zijn er twee medicamenteuze opties, namelijk peginterferon of RBV. Vanwege het bijwerkingenprofiel wordt er veelal gekozen voor RBV. RBV wordt meestal toegediend in een dosering van 600–800 mg per dag, gedurende 3 maanden, waarbij een succespercentage van 63% is beschreven. Verder is beschreven dat, als HEV-RNA in faeces nog aantoonbaar is aan het einde van de behandeling, de kans op therapie-falen groot is.
Bevindingen
Dit proefschrift beschrijft verschillende klinische aspecten van virale hepatitis B, C en E. We beschrijven dynamische veranderingen in de immuunrespons tijdens acute hepatitis B. Daarnaast onderzoeken we of intrahepatische en plasma IP-10 spiegels een responsmarker zijn voor interferon-bevattende therapie bij chronische hepatitis B en C virus infectie. Verder beschrijven we enkele behandelopties voor chronische hepatitis B, C en E virus infectie en proberen we middels predictiemodellen een voorspelling te doen over de hepatitis C-gerelateerde ziekte last in 2030. Tot slot bestuderen we de prevalentie van chronische hepatitis E virus infectie onder patiënten met een allogene hematopoetische stamceltransplantatie en de klinische relevantie van een dergelijke infectie in deze groep patiënten.

Deel I - Hepatitis B Virus Infectie
Hoofdstuk 1 beschrijft de immuunrespons van 9 patiënten met een acute HBV-infectie, en dan met name de NK-cel karakteristieken en de HBV-specifieke T-cel functie. Eén van deze 9 patiënten ontwikkelde een chronische HBV-infectie terwijl de andere 8 patiënten genazen van de infectie. Vroeg na de HBV infectie zagen we een toename van het totaal aantal CD56<sup>bright</sup> NK-cellen, en een toename van het deel van deze cellen die activatiemarkers tot expressie brengen. De meeste hiervan normaliseerden na klaren van de virusinfectie, terwijl bij de patiënt met chronische HBV-infectie het aantal TRAIL-positieve CD56<sup>bright</sup> NK-cellen hoog bleef. Bij de patiënten die HBV klaarden, zagen we een functionele HBV-specifieke CD4+ en CD8+ T-cel respons, maar bij de patiënt die chronische infectie ontwikkelde zagen we dit niet. Dit wijst erop, dat NK-cellen vroeg tijdens een acute infectie met HBV geactiveerd raken. Patiënten die een acute infectie met het HBV klaren, hebben een brede, en multi-specifieke T-cel respons. Zoals geïllustreerd door de patiënt die een chronische HBV-infectie ontwikkelt, zou het niet afnemen van NK-cel activatie en de ontwikkeling van een zwakke T-cel respons kunnen verklaren waarom de infectie chronisch is geworden.

In Hoofdstuk 2 hebben we gekeken naar de rol van IP-10 plasma spiegels vóór en tijdens de behandeling met peginterferon en adefovir van chronische hepatitis B (CHB) patiënten met een hoge concentratie van hepatitis B virusdeeltjes. We vonden in deze studie dat plasma IP-10 spiegels en IP-10 mRNA expressie in de lever vóór start van de behandeling aan elkaar gerelateerd zijn, vooral bij HBeAg-positieve CHB patiënten. Hogere plasma IP-10 spiegels vóór start van behandeling waren geassocieerd met een hogere kans op een gecombineerde respons (verlies van HBeAg, normalisatie van ALAT en HBV-DNA onder 2000 IU/mL). Dit zagen we bij HBeAg-positieve CHB-patiënten, maar niet bij HBeAg-negatieve CHB-patiënten. Verder vonden we een positief verband tussen plasma- en intra-hepatische IP-10 spiegels. Daarnaast was plasma IP-10 gecorreleerd met het gehalte aan ALAT en HBV-DNA in het plasma, en met HAI score bij leverbiopsie.

Hoofdstuk 3 beschrijft een gerandomiseerde gecontroleerde studie bij CHB patiënten met een laag aantal virusdeeltjes in het bloed, die werden behandeld met combinatietherapie bestaande uit peginterferon en adefovir of peginterferon en tenofovir. Daarnaast was er een controle groep die geen behandeling ontving. De hoofdvraag was, of er vaker functionele genezing op zou treden bij patiënten die behandeld werden met combinatietherapie in vergelijking met de controlegroep. In deze studie vonden we
geen significant hoger percentage HBsAg-verlies onder patiënten die met combinatietherapie werden behandeld ten opzichte van patiënten die niet behandeld werden. We vonden wel vaker een sterke HBsAg-daling van > 1log10 IU/mL bij 21% van de patiënten behandeld met combinatietherapie, ten opzichte van geen daling van HBsAg bij patiënten die niet behandeld werden.

Deel II - Hepatitis C Virus Infectie
In Hoofdstuk 4 beschrijven we dynamische veranderingen in plasma IP-10 bij chronische hepatitis C (CHC) patiënten vóór en tijdens behandeling met hoge inductie dosis standaard interferon (IFN) gedurende zes weken, gevolgd door een geregisteerde dosis pegIFN en RBV waaraan amantadine was toegevoegd. We konden geen significante relatie tussen IP-10 plasma-spiegels vóór of tijdens behandeling, en succes van de behandeling (sustained virological response, SVR) aantonen. Wel zagen we dat IP-10 spiegels op Dag 1 na start van de hoge dosis inductietherapie met interferon sterk waren gestegen vergeleken met de spiegels vóór de behandeling. De stijging in IP-10 spiegel op dag 1 was gecorreleerd met een daling in HCV-RNA van >2.28log10 IU/mL op datzelfde tijdstip. Ook was een sterke stijging van IP-10 op dag 1 gecorreleerd met een gunstig IL28B genotype. Daarnaast zagen we dat de mate van stijging van IP-10 plasma-spiegels op Dag 1 van de behandeling afhankelijk was van de IP-10 spiegel vóór start van de behandeling (basis-spiegel). Bij patiënten met een lage IP-10 basis-spiegel van <150 pg/mL werd een bijna 30-voudige stijging gezien op Dag 1 van de behandeling, terwijl bij patiënten met een hoge IP-10 basis-spiegel van >600 pg/mL slechts een viervoudige stijging werd waargenomen.

Hoofdstuk 5 beschrijft de resultaten van een retrospectieve observatiele “real-life” cohort studie in de regio Amsterdam onder patiënten met chronische hepatitis C (CHC) genotype 4 en gevorderde leverfibrose of gecompenseerde levercirrose. Deze patiënten werden behandeld met een combinatie van sofosbuvir en simeprevir met of zonder RBV. Bij 49 van de 53 (92%) patiënten was de behandeling succesvol (er trad een blijvend virale respons op). Dit percentage is vergelijkbaar met dat uit grote registratietudies voor de combinatie van sofosbuvir en simeprevir bij patiënten met CHC genotype 1. Of de toevoeging van RBV bij patiënten met (gecompenseerde) levercirrose de kans van slagen van deze behandeling vergroot, wordt niet duidelijk uit onze studie. In ons cohort hadden alle 12 patiënten, waarbij RBV was toegevoegd aan sofosbuvir en simeprevir, een blijvende virale respons, terwijl bij de 4 patiënten, die een niet-succesvol verlopen behandeling hadden, allen behandeld waren zonder de toevoeging van RBV.

In Hoofdstuk 6 wordt de ziektegeschiedenis beschreven van een patiënt met CHC genotype 1a en gevorderde leverfibrose. In het verleden had zij eerst een niet-succesvolle behandeling met pegIFN en RBV. Vervolgens had zij een niet-succesvolle behandeling met een combinatie van een proteaseremmer (NS3) en een NS5A-remmer in onderzoeksverband, waarbij ze een RAS in het NS3 en NS5A gebied ontwikkeld. Anderhalf jaar na deze behandeling was de RAS in het NS3 gebied niet meer aantoonbaar maar die in het NS5A gebied nog wel. Omdat patiënt ook ernstig overgewicht had, onderging zij in de wachtijd voor registratie en vergoeding van de nieuwe DAA’s, bariatrische chirurgie (een gastric bypass met Roux-en-Y reconstructie). Na het beschikbaar komen van de verschillende DAA’s kon patiënte succesvol behandeld worden met sofosbuvir, simeprevir en RBV gedurende 24 weken. Echter, door de bariatrische chirurgie was er suboptimale
opname van simeprevir, wat zich uitte in simeprevir plasma-spiegels die sub-therapeutisch waren vergeleken bij wat in eerdere literatuur beschreven was. Derhalve werd de dosis van simeprevir verdubbeld, wat leidde tot adequate plasmaspiegels. De gemeten plasma-spiegels van sofosbuvir en RBV waren adequaat. Deze casus illustreert het nut van “therapeutic drug monitoring” (TDM) in dergelijke speciale patiëntengroepen.

In Hoofdstuk 7 wordt een voorspelling gedaan middels modelleren, wat het effect van verschillende behandelingstrategieën is op het toekomstige aantal patiënten in 2030 met CHC en de daarmee samenhorende ziektelast (gedefinieerd als het optreden van lever-gerelateerde mortaliteit en de incidentie van HCC). De belangrijkste scenario’s die gebruikt zijn om dit te berekenen zijn:

A. “basis-scenario”, waarbij CHC patiënten met ernstige fibrose of cirrose (META-VIR ≥ F3/F4) behandeld worden met interferon-bevattende therapie;
B. “toegenomen effectiviteit”, waarbij CHC patiënten met ernstige fibrose of cirrose (META-VIR ≥ F3/F4) behandeld worden DAA’s;
C. “alle CHC patiënten”, waarbij alle CHC patiënten worden behandeld met DAA’s, onafhankelijk van fibrose stadium;
D. “screening en eliminatie”, waarbij alle patiënten worden behandeld met DAA’s onafhankelijk van fibrose stadium, maar dan uitgebreid met het aantal nieuw gediagnosticeerde CHC patiënten, waarbij tevens rekening gehouden werd met een lager aantal acute HCV-gevallen door preventiemaatregelen.

De prevalentie van CHC in Nederland in 2013 was 0,12 %. Dit is de prevalentie die is gebruikt voor het berekenen van de scenario’s. In het basis-scenario zou het aantal HCV-geïnfecteerden in 2030 met 45% afnemen ten opzichte van 2013/2014. Het aantal patiënten met HCC en de lever-gerelateerde mortaliteit zou in dat geval dalen met respectievelijk 19% en 27%. Als het aantal behandelde patiënten uitgebreid zou worden naar alle leverfibrose stadia (scenario C), dan zou het aantal HCV-geïnfecteerden naar schatting met 85% dalen. Het aantal patiënten met HCC en de lever-gerelateerde mortaliteit zou dan dalen met 60–68%. Ook bij de andere beschreven scenario’s zou er een significante daling zijn in het aantal CHC patiënten, in het optreden van HCC, en in de lever-gerelateerde mortaliteit.

Deel III - Hepatitis E Virus Infectie

In Hoofdstuk 8 beschrijven we de prevalentie van chronische HEV-infectie in een cohort patiënten die een allogene hematopoïetische stamceltransplantatie (alloHSCT) hebben ondergaan in Amsterdam van 2005–2015. Chronische HEV-infectie werd vastgesteld bij 5 van de 130 patiënten (4 %), wat hoger is dan eerder was beschreven in Nederland bij andere groepen immuungecompromitteerde patiënten, zoals ontvangers van een solide orgaantransplantatie en HIV-geïnfecteerde patiënten (0.12–3%). Er zou een relatie kunnen zijn tussen chronische HEV-infectie en hepatische GvHD aangezien er bij drie van de vijf patiënten met chronische HEV-infectie een sterke verdenking op hepatische GvHD bestond. In twee van deze patiënten leidde behandeling met ribavirine tot zowel het klaren van de HEV-infectie als het verdwijnen van GvHD. Bij de derde patiënt was de HEV-infectie niet eerder gediagnosticeerd omdat de patiënt ten tijde van het vaststellen van de HEV-infectie (retrospectief) al overleden was. De HEV-infectie in deze patiënt is daarom onbehandeld gebleven. GvHD in deze patiënt was
therapie-refractair. Concluderend kunnen we zeggen dat we een hogere prevalentie (4%) chronische HEV-infectie vonden bij patiënten met een alloHSCT, dan is beschreven bij andere groepen immuungecompromitteerde patiënten. Daarnaast zouden onze bevindingen kunnen wijzen op een relatie tussen hepatische GvHD en chronische HEV-infectie.
### LIST OF ABBREVIATIONS

- **alloHSCT** – allogeneic Hematopoietic Stem Cell Transplantation
- **ALT** – Alanine Transferase
- **Anti-HBc** – Anti-Hepatitis B core antibodies
- **Anti-HBe** – Anti-Hepatitis B e antibodies
- **Anti-HBs** – Anti-Hepatitis B surface antibodies
- **APC** – Antigen Presenting Cell
- **CHB** – Chronic Hepatitis B
- **CHC** – Chronic Hepatitis C
- **DAA** – Direct-acting Antiviral Agent
- **DNA** – DesoxyNucleic Acid
- **E1** – Envelope 1
- **E2** – Envelope 2
- **EMA** – European Medicines Agency
- **FLF** – Fulminant Liver Failure
- **GvHD** – Graft versus Host Disease
- **HBcAb** – Hepatitis B core Antibodies
- **HBcAg** – Hepatitis B core Antigen
- **HBeAg** – Hepatitis B e Antigen
- **HBeAb** – Hepatitis B e Antibodies
- **HBsAg** – Hepatitis B surface Antigen
- **HBsAb** – Hepatitis B surface Antibodies
- **HBV** – Hepatitis B Virus
- **HCC** – Hepatocellular Carcinoma
- **HCV** – Hepatitis C Virus
- **HEV** – Hepatitis E Virus
- **HIV** – Human Immunodeficiency Virus
- **IFN** – Interferon
- **IL** – Interleukin
- **IL28B** – Interleukin 28 B
- **IP-10** – Interferon-gamma inducible Protein 10
- **ISG** – Interferon Stimulated Gene
- **MSM** – Men who have Sex with Men
- **mRNA** – messenger RiboNucleic Acid
- **NA** – Nucleo(s)tid Analogue
- **NK-cell** – Natural Killer cell
- **NS** – Non-Structural
- **ORF** – Open-Reading Frame
- **Peg** – polyethylene glycol
- **PegIFN** – Peginterferon
- **PWID** – People Who Inject Drugs
- **RAS** – Resistance-Associated Substitutions
- **RBV** – Ribavirin
- **RNA** – RiboNucleic Acid
- **SNP** – Single Nucleotide Polymorphism
- **SOT** – Solid Organ Transplant
- **SVR** – Sustained Virological Response
- **TNF** – Tumor Necrosis Factor
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LIST OF PUBLICATIONS

IP-10 in chronic hepatitis C patients treated with high-dose interferon.

Historical epidemiology of hepatitis C virus (HCV) in select countries – volume 2.

The present and future disease burden of hepatitis C virus (HCV) infections with today's treatment paradigm - volume 2.

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*Bone Marrow Transplant* 2017;52(4):622-624.

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*Antiviral Res* 2017;146:139-45.

The observed effect of gastric bypass surgery on the treatment of chronic hepatitis C virus (HCV) infection; A case report.  
Smolders EJ, Willemse SB, El-Sherif O, Khoo S, Burger D.  

Dynamics of the immune response in acute hepatitis B infection.  
*In press Open Forum for Infectious Diseases* 2017.
Low compliance with hepatocellular carcinoma screening guidelines in hepatitis B/C virus co-infected HIV-patients with cirrhosis.


Submitted

Richtsnoer behandeling hepatitis C virus infectie in Nederland.


Available at: http://www.hcvrichtsnoer.nl
## PHD PORTFOLIO

**Name PhD student:** Sophie Bertine Willemse  
**PhD period:** 1st May 2012 – 1st January 2017  
**Name PhD supervisors:** U.H. Beuers, H.L. Zaaijer, H.W. Reesink and M. van der Valk

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<td>– Teach the teacher (&quot;coach the intern&quot; and &quot;training of fellows&quot;)</td>
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<td>– Weekly Gastroenterology and Hepatology seminars</td>
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<td>– HCV Preceptorship Lyon</td>
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<td>– Paris Hepatitis Conference</td>
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<td>– United European Gastroenterology Week (UEGW)</td>
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<td>– EASL, Amsterdam, NL: Serum IP10 levels at baseline and during therapy do not predict SVR in CHC patients treated with high induction dose interferon.</td>
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<td>– AASLD, Washington, USA: T-Cell Characteristics during Acute Hepatitis B Infection</td>
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<td>– AASLD, Boston, USA: Lack of compliance to hepatocellular carcinoma screening guidelines in hepatitis B or C virus co-infected with HIV patients with cirrhosis</td>
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## Appendices

### 1. Oral Presentations

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### 2. Teaching

#### Lecturing

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<td>Dutch Liver Week 2014: Viral hepatitis – clinical cases</td>
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#### Tutoring, Mentoring

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<td>Bachelor Thesis Robin Erken: HEV-infection</td>
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#### Supervising

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<th>Year</th>
<th>ECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fellows in training: 3-5 per year</td>
<td>2012-2017</td>
<td>3.0</td>
</tr>
<tr>
<td>Research fellows: management of clinical research patients</td>
<td>2012-2017</td>
<td>3.0</td>
</tr>
</tbody>
</table>

### 3. Other

<table>
<thead>
<tr>
<th>Role</th>
<th>Year</th>
<th>ECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Member of HEPNED</td>
<td>2015-2017</td>
<td>1.2</td>
</tr>
<tr>
<td>Dutch HCV Richtsnoer Committee (National HCV Guidelines committee)</td>
<td>2015-2017</td>
<td>1.5</td>
</tr>
</tbody>
</table>
DANKWOORD

Had men mij 10 jaar geleden gevraagd of ik ooit zou promoveren, had ik daar ongetwijfeld “nee” op geantwoord. Het ging heel anders... Zonder de invloed, hulp, en steun van velen was dit boekje er nooit gekomen. Hier is dan eindelijk de gelegenheid om jullie allemaal te bedanken.

Allereerst wil ik mijn promotores en co-promotores bedanken. Wat begon als een tweekoppige eenheid, verdubbelde zich in de ontknoping. Ulrich, bij onze eerste ontmoeting werd al snel duidelijk dat wij meerdere liefdes delen, zoals skiën, wijn, gezelligheid, en de lever. Dat mijn plek in het AMC was, werd in de loop der jaren steeds duidelijker. Op jouw aangeven kreeg dat meer kleur en heroverwoog ik om alsnog te gaan promoveren. Vele uitstapjes en skiweekenden later is hier dan toch eindelijk een seriaal resultaat: dit werk. Dank je voor je vertrouwen en de kans die je me bood.

Henk, het is indrukwekkend hoe jij als een “mammoet-tanker” altijd maar doorgaat tot je doel bereikt is. Je kritische, scherpe blik en enorme ervaring maken dat je als geen ander vooruit ziet hoe projecten opgezet dienen te worden, en wat nut heeft om opgeschreven te worden. Hoe blij was ik dan ook toen je je de keer ongelijk had, en een van mijn stukken tegen jouw verwachtingen in “zomaar” geaccepteerd was. Ik waardeer je toegankelijkheid, persoonlijke betrokkenheid en je karakteristieke cynische noot enorm. Bedankt dat je mij de kans bood om naast mijn klinische werk bij jou te promoveren.

Hans, vanaf mijn eerste schreden op onderzoeksgebied hebben onze paden elkaar gekruist. Ik wilde lijst na lijst samples uitgevuld hebben door een van jouw analisten, maar jij wilde eerst het protocol maar eens zien. Eerste les geleerd: zonder protocol geen materiaal en dus geen onderzoek... Jaren later dachten we samen met Henk na over leuke nieuwe HEV-projecten, en overal bleek wel weer een klein lesje van “ome Hans” in te zitten. Ik bewonder je gave om aan de ene kant luchtig en laagdrempelig te zijn, maar aan de andere kant toch ook een streng leraar. Bedankt je dat je mijn promotor wilt zijn.


Geachte promotiecommissie, veel dank voor het kritisch beoordelen van mijn proefschrift en het plaatsnemen in mijn promotiecommissie.

Paul, ook jij staat in vele opzichten aan de basis van dit boek. Jij nam me aan voor de opleiding, en maakte het vervolgens mogelijk om als promoverend staflid te blijven in het AMC. Je positieve, relaxte maar waar nodig daadkrachtige manier van leidinggeven maakt je een prettige baas en een voorbeeld. Veel dank voor je vertrouwen en je plaats in mijn promotiecommissie.

Peter, mijn eerste stappen in het AMC als oudste co-assistent en beginnend onderzoeker begeleidde jij. Toen ik na een jaar met mijn opleiding begon, was er tot jouw grote teleurstelling voor mij geen ruimte voor onderzoek. Ik leerde jouw directe manier
van communiceren kennen, iets wat ik pas jaren later echt op waarde wist te schatten. Jouw visie en ideeën over onderzoek zijn complex en inspirerend, en je provocerende opmerkingen en vragen leiden tot geanimeerde, en soms hilarische discussies. Ik ben je dankbaar dat je plaats wilt hebben in mijn promotiecommissie. En ik beloof je, het endotheline-verhaal wordt echt nog opgeschreven!


Joanne, een leukere en betere leverpatholoog bestaat niet! De leverpathologie-besprekingen zijn elke vrijdag weer een feest, en ik hoop nog lang met je te mogen samenwerken. Dank dat je plaats hebt willen nemen in mijn promotiecommissie.

Prof. dr. Bart van Hoek en prof. dr. Rob de Man, Beste Bart en beste Rob, ik ben vereerd met jullie kritische blik en plaats in mijn promotiecommissie, veel dank hiervoor.

Beste co-auteurs, bedankt voor jullie inspanningen en de prettige samenwerking.

Ook wil ik de patienten bedanken die aan de studies hebben deelgenomen, waaruit de studies beschreven in dit proefschrift voortgevloeid zijn.

Over de jaren zijn er veel verschillende collega’s die mijn pad gekruist hebben. Het zijn er teveel om op te noemen, dus ook aan iedereen die ik vergeet: bedankt!

Christine, onder jouw bezielende leiding leerde ik hoe leuk het is om patiënten met virale hepatitis te behandelen, maar ook hoe veel bijwerkingen interferon heeft. Je was en bent een voorbeeld en je gaf om van elke saaie bijeenkomst een kleurrijk feestje te maken is bewonderenswaardig. Huub, jij was destijds de ervaren rot wat betreft hepatitis-onderzoek. Nog voor een eerder project goed en wel gestart was, kwam jij steeds weer met een nieuw ander briljant (on)uitvoerbaar idee. Helaas zit je aan de andere kant van de wereld, maar ik hoop je gauw weer tegen te komen. Joep, ik heb je vooral meegemaakt als AIOS MDL, maar mijn eerste artikel komt uit jouw koker. Dank voor je aanstekelijke humor en de prettige samenwerking.

Bart, als jonkies begonnen wij vol frisse moed met onderzoek bij de hepatologie als kamergenoten, en na vele opleidings-omzwervingen zijn we opnieuw kamergenoten als staflid. Al breek ik dagelijks bijna m’n nek over jouw rondslingerende spullen, een betere roomie kan ik me niet wensen. Dank je voor je vriendschap, humor en gezelligheid.

Martine, Jeltje, en Hadassa, jullie zijn al jaren de stabiele basis voor de hepatitis-research groep en de patienten die aan alle onderzoeken deelnemen. Niets is jullie teveel. Dank voor jullie enthousiasme, hulp en ondersteuning. Ruth, onze eerste ontmoeting was toen Peter mij aan jou voorstelde op de oude hepa-pol. “Het is een meisje” riep je vol enthousiasme. En dat riep ik vorig jaar ook tegen jou toen ik zwanger was. Dank voor je steun en interesse, en dat je mij vaak even tot de orde roept als ik het “te” druk heb. Frank, ook jij bedankt voor de prettige samenwerking op de poli, en hopelijk nog vele jaren te komen! Marjan, jij leerde me geduldig alles over FACS-en en PBMC's isoleren. Helaas bleek het toch niet te combineren: werken op het lab en een klinische baan. Jij bleef echter altijd een even hulpvaardige steun en toeverlaat. Gelukkig ben je in de buurt op een goede plek terecht gekomen.

Collega stafleden MDL in het AMC, leukere collega's kan ik me niet wensen. We zijn een lekker stel bij elkaar, allemaal op onze eigen manier gedreven en doelbewust, maar toch ook persoonlijk bij elkaar betrokken. Zolang we elkaar niet uit het oog verliezen, komt het allemaal wel goed. Manon, jij had het niet verwacht (ik daarentegen wel…), maar nu ga jij toch echt eerst promoveren! Echt supergoed van je! Maatjes in het Slotervaart (onder de bezielende leiding van Annekatrien), nu al meerdere jaren samen in het AMC, ik hoop dat dat nog heel lang door mag gaan! Jonge staf, veel dank voor de gezelligheid, roddels, en luisterende oren. Assistenten (en oud-assistenten) MDL, het is (en was) elke dag een feest om met jullie samen te werken. Endoscopie-verpleegkundigen, baliemedewerkers van poli en endoscopie, verpleegkundigen van F7 Zuid, collega's internisten, radiologen en chirurgen, allen dank voor de fijne samenwerking. En ook het secretariaat MDL: Monique, Sherille, Linda, Karina en Patricia, veel dank voor jullie ondersteuning.

Lieve vrienden van tennis, dispuut en cluppie, ook al heb ik jullie de afgelopen tijd om de welbekende redenen veel minder gezien, ik waardeer jullie niet minder. Dat geldt ook voor jou, lieve Nies, we zien elkaar niet vaak, maar het is altijd goed. Ook nu we elkaar van een afstand volgen nu jij in de VS zit. Veel dank allemaal voor alle gezelligheid en steun.

Lieve paranimfen, lieve vriendinnetjes, wat bijzonder en fijn, en wat een eer dat jullie mij terzijde zullen staan.

Mir, onafscheidelijk waren wij in Groningen: wijn drinken, stappen, taco's eten, dispuut. Jij, een jaar ouder, ging me in alles een jaartje voor. Promoveren doe ik echter wel iets later dan slechts één jaar na jou… Ik ben blij dat we elkaar weer gevonden hebben, en dat je wat dichter bij woont.

Liek, in 2005 kwam ik bij jullie in het tennisteam, en dat was een gouden greep. Eerst samen met Jaco en Eline, maar over de jaren bleven wij met z'n tweeën over, en deelden naast veel gezelligheid, ook lief en leed. Twee handen op een buik, en toch kunnen we samen niet dubbelen...

Lieve familie Willemse, Becker, Kuijpers, Van de Sandt, Koppen en Post, jullie kleine nichtje promoveert! We zien elkaar niet vaak, maar leven niet minder met elkaar mee. Veel dank voor jullie interesse, steun en betrokkenheid.

Piet, mijn lievelingsoom. Jij kijkt al jaren reikhalzend uit naar mijn promotie, en hoewel ik twijfelde of het ooit ging gebeuren is het mede door jou, én voor jou, gelukt! Jij bent mijn grootste fan, en ik de jouwe. Dit boek is niet voor niets aan jou opgedragen.
Matthijs, enorm bedankt voor het ontwerp van de omslag van dit boek. Wat heerlijk en bijzonder, dat jij, mijn neef, dit kan en doet. Het boek is er extra mooi van geworden! Stefan, das Studio-genoot van Matthijs, jij ook bedankt voor je gedetailleerde opmaak van het boek.

Dear Alex, Tim, Taitum, Alba and Taner, my family at the other side of the world. It is hard to be so far apart from your family, but you guys never leave my mind. Now this work is finished, I hope I can see you more often via facetime, and hopefully I won’t procrastinate so much on booking holidays...

Lieve pappa en mamma, jullie onvoorwaardelijke steun en liefde is hartverwarmend en ik kan jullie niet genoeg bedanken. Na een kleindochter begin dit jaar, komt er nu iets wat in jullie enorme boekenkast past. Het jaar 2017 is een goed jaar...

Lieve Isabelle, jij bent de mooiste en de liefste van de hele wereld.

Finally, my love Reuben, my king of hearts. You are my driving force. Without you, I would never have finished this book. The future is ours.
CURRICULUM VITAE

Sophie Willemse was born in Saint-Germain-en-Laye, France, on September 18, 1979. She grew up in Hilversum, the Netherlands, and graduated at the Gemeentelijk Gymnasium in 1997. During the same year, she began medical studies at the University of Groningen, and obtained her medical degree in 2005. At the end of her medical studies, she did research for her master thesis on occult hepatitis B infection in patients with chronic hepatitis C virus infection, under supervision of prof. dr. P. Marcellin of Beaujon Hospital, Paris, France. It is during that time that she became interested further in Hepatology, and in particular, in viral hepatitis. After working as a research fellow for a year at the department of Hepatology, she began her medical specialist training in Gastroenterology and Hepatology at the at the Academic Medical Centre (AMC), Amsterdam, the Netherlands, in 2006, with parts of the training at the Onze Lieve Vrouwe Gasthuis, and the Slotervaart Ziekenhuis, both in Amsterdam, and at the Liver Unit of Beaujon Hospital in Paris. At the end of her training in 2012, she remained as a staff member at the department of Gastroenterology and Hepatology of the AMC with special focus on Hepatology. During this time, next to her work as a clinician in Gastroenterology and Hepatology, she also was a PhD student under supervision of dr. H.W. Reesink and prof. dr. U. H. Beuers at the department of Gastroenterology and Hepatology, prof. dr. H. L. Zaaijer at the department of Clinical Virology and dr. M. van der Valk at the department of Internal Medicine, Division of Infectious Diseases. Her research is focused on clinical aspects of hepatitis B, C, and E virus infection, and the results of which have culminated towards this thesis.
Sophie Willemse

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